

ALTERATIONS IN CALCIUM HOMEOSTASIS AS A
POSSIBLE CAUSE OF OCHRATOXIN A NEPHROTOXICITY

CENTRE FOR NEWFOUNDLAND STUDIES

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XIN CHONG

ALTERATIONS IN CALCIUM HOMEOSTASIS AS A POSSIBLE CAUSE OF
OCHRATOXIN A NEPHROTOXICITY

by

©Xin Chong, B.Sc

A thesis submitted to the School of Graduate
Studies in partial fulfilment of the
requirements for the degree of
Master of Science

Biochemistry

Memorial University of Newfoundland

St. John's, Newfoundland

February, 1993



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ABSTRACT

The carcinogenic nephrotoxin Ochratoxin A (OTA) is a widespread natural contaminant of a variety of food and feedstuffs. The effect of OTA on renal lipid peroxidation and on renal cortical endoplasmic reticular ATP-dependent calcium uptake were investigated.

OTA did not stimulate NADPH-dependent lipid peroxidation (malondialdehyde formation) in the kidney, either in vivo or in vitro. It also did not induce any of several antioxidative enzymes in vivo under our experimental conditions. Administration of a single high dose or multiple lower doses of OTA to rats did result in an increase of the renal cortical endoplasmic reticular ATP-dependent calcium pump activity. This increase was very rapid, being evident within 10 minutes of OTA administration, and remained elevated for at least 6 hours thereafter. This change in calcium uptake is the earliest enzymatic change reported in the kidney after OTA administration.

In vitro preincubation of microsomes with NADPH had a profound inhibitory effect on calcium uptake. Inclusion of OTA was able to reverse the inhibition. Addition of OTA alone to cortical microsomes during calcium uptake inhibited the uptake process, although the effect was reversible. However, it does not appear that in vivo OTA exerts a significant

direct inhibitory effect on renal cortical calcium uptake activity since an increase in this activity was observed after OTA administration.

Changes in the rates of microsomal calcium uptake were correlated with changes in the steady-state levels of the phosphorylated Mg^{2+}/Ca^{2+} -ATPase intermediate suggesting that in vivo/in vitro conditions were affecting the rate of enzyme phosphorylation.

Study of OTA analogues indicated that the presence of a free carboxyl group and chlorine atom on OTA contributed significantly to the effect on calcium uptake.

These results suggest that lipid peroxidation may not be responsible for the initiating OTA nephrotoxicity. However, alterations in calcium homeostasis as evidenced by an increase in microsomal calcium pump activity could play a significant role in OTA nephrotoxicity.

To
My Parents

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LIST OF ABBREVIATIONS

CDNB	1-chloro-2,4-dinitrobenzene
DCPIP	2,6-dichlorophenol indophenol
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid disodium
ER	endoplasmic reticulum
GSH	reduced glutathione
GSSG	oxidized glutathione
hr	hour(s)
MDA	malondialdehyde
MFO	mixed function oxidase
min	minute(s)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
OT α	ochratoxin α
OTA	ochratoxin A
OTB	ochratoxin B
OTC	ochratoxin C
Phe	phenylalanine
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid

C H A P T E R 1

1. INTRODUCTION

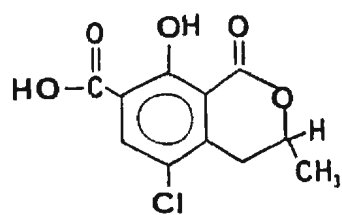
1.1 Ochratoxin A

1.1.1 Sources, occurrence and properties

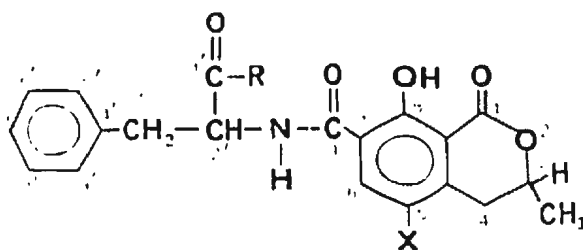
The ochratoxins encompass a group of closely-related secondary metabolites of the fungi Penicillium and Aspergillus. These fungi are ubiquitous and the potential for contamination of foodstuffs and animal feed is widespread. Ochratoxin A (OTA), the major metabolite and the most toxic of the ochratoxins, has been found in a number of countries worldwide, mainly in Europe. Ochratoxins formation by Aspergillus species appears to be limited to conditions of high humidity and temperature, whereas at least some Penicillium species may produce ochratoxin at temperatures as low as 5°C [WHO, 1990].

Following the discovery of ochratoxins by De Scott in 1965, OTA, the 7-carboxy-5-chloro-8-hydroxy-1,4-dihydro-3-R-methylisocoumarin amide of L- β -phenylalanine, was isolated together with its less toxic analogue, ochratoxin B (OTB) (dechloro-ochratoxin A) from a culture of Aspergillus ochraceus [Merwe et al, 1965a&b]. The ochratoxin group (Fig. 1) consists of OTA; OTB; methyl ester of OTA,

Fig. 1. The chemical structure of ochratoxins



Ochratoxin α (OT α)



Ochratoxin A: X = Cl; R₁ = H

Ochratoxin B: X = H; R₁ = H

Ochratoxin C: X = Cl; R₁ = CH₃

ochratoxin C (OTC); 4-hydroxyochratoxin A; hydrolysed OTA (isocoumarin part), ochratoxin α ; methyl ester of OTB; methyl and ethyl esters of OTA. OTA and, very rarely, OTB are the only compounds found as natural contaminants in plant material, and most information available concerns OTA [Who, 1990]. OTA is very stable in ethanol at 0-4°C; however, such solutions are light sensitive, since decomposition occurs on exposure to fluorescent light for several days. OTA is a colourless, crystalline compound, obtained by crystallization from benzene, with a melting point of about 90°C. It is soluble in polar organic solvents, slightly soluble in water, and soluble in dilute aqueous bicarbonate. OTA is also optically active.

Both OTA and OTB have been found as natural contaminants of food, feed and their products, though OTB occurs extremely rarely. Agricultural commodities in which OTA has been detected include corn, wheat, rye, hay, barley, oats, sorghum, groundnuts, coffee beans, etc. at levels ranging from 5 - 27,500 $\mu\text{g/Kg}$ [WHO, 1979]. The levels vary significantly from country to country. In Canada, Scott et al [1972] detected OTA in concentrations of up to 27 ppm in 18 out of 29 samples of heated grain from Saskatchewan farms. Abrahamson et al [1983] also showed 7.8% of mixed animal feed from western Canada was contaminated with OTA at levels ranging from 48 - 5900 $\mu\text{g/Kg}$.

Residues of OTA are not generally found in ruminants, because OTA is cleaved in the forestomach by protozoan and bacterial enzymes [Galtier & Alvinerie, 1976; Hult et al., 1976; Patterson et al., 1981]. The non-toxic acid hydrolysate of OTA, OT α , is a cleavage product found in the intestine, feces, urine, kidneys, and liver of rodents experimentally fed an OTA-containing diet [Galtier & Alvinerie, 1976]. Residues of OTA have been detected in a number of tissues in single-stomach animals, such as the pig. Data on levels of OTA [Roschenthaler et al, 1984] indicate that when contamination of feed by OTA is significant, the mycotoxin can occur commonly in swine kidney and liver, in smoked meat products, and in the blood of slaughtered swines. These data indicated that 83% of the samples contained <200 μ g/Kg OTA, but a few contained the level of OTA as high as 920 μ g/Kg. The highest levels of OTA residues were normally found in kidneys with lower levels in the liver, muscle, and adipose tissue [Krogh et al, 1974]. OTA is present in the blood bound to serum-albumin [Chu, 1971; Galtier, 1974a&b] and as free OTA; saturation (in the rat) occurs at 70 mg OTA/L plasma. In Western Canada, it was reported that 3.6 - 4.2% of 1200 pig blood samples, collected from slaughterhouses during 1986, contained OTA at concentrations higher than 20 ng/mL [Marquardt et al, 1988]. The binding of OTA to serum albumin is particularly strong in cattle, pigs, and man, based on in

vitro studies [Galtier, 1979]. Chu showed that serum albumin bound two equivalents of OTA tightly [1971].

The potential for human exposure to OTA is very high because of the consumption of contaminated food. Recently, OTA has been detected in the blood of 6-18% of the human population in some areas where Balkan endemic nephropathy is prevalent [WHO, 1990]. OTA has also been found in human blood samples outside the Balkan peninsula. In some studies, more than 50% of the samples analyzed were contaminated [WHO, 1990]. A high incidence of tumours of the urinary system is strongly correlated with the prevalence of Balkan endemic nephropathy [Krogh, 1977, Krogh et al 1977]. The frequency of occurrence of this disease is correlated with relatively high concentrations of OTA in the food from endemic regions [Krogh, 1977] and with the concentration of OTA in human blood [Hult et al, 1982]. However, a direct causal relationship cannot be established on the basis of indirect evidence provided by the above retrospective studies alone. Neither can it be excluded in view of the long latency period between the exposure and the onset of symptoms.

1.1.2 Biological and biochemical activity

OTA acts as a potent nephrotoxin in all animals investigated so far, including chickens, dogs, ducklings,

mice, rats, hens, sheep, swine and rainbow trout [WHO, 1979]. Different species vary in their susceptibility to acute poisoning by OTA with LD₅₀ values ranging from 3.4 to 34.3 mg/Kg (Table 1).

When OTA was administered orally to rats, the kidney was the target organ, but necrosis of periportal cells in the liver has also been noted during studies on acute effects [Purchase & Theron, 1968].

The toxic effect of OTA is demonstrated initially on the nephron; the proximal tubule is the primary target site. Changes of renal function on OTA-exposed pigs and rats are characterized by impairment of proximal tubular function, indicated by a decrease of urine osmolality, and by increased urinary excretion of proteins and glucose [Berndt et al, 1980]. Based on the reported inhibition of transport of p-aminohippurate (PAH) and tetraethylammonium in renal slices, it was proposed that OTA enters the proximal tubule cells by the common organic anion transport system [Sokol et al, 1988]. A particularly good correlation exists between the increase in the urinary excretion and decrease in renal activity of γ -glutamyl transferase (γ -GT), alkaline phosphatase (ALP) and leucine aminopeptidase (LAP) within a week after oral administration of 145 μ g OTA/Kg body weight (bw)/day for 12 weeks [Kane et al, 1986a]. Inhibition of gluconeogenesis was

Table 1

Acute toxicity of Ochratoxin A

Animal	LD ₅₀ (mg/Kg bw)	Route of administration
mouse (female)	22	intraperitoneal (i.p.)
rat (male)	30.3	oral
rat (female)	21.4	oral
rat (male)	28	oral
rat (male)	12.6	i.p.
rat (female)	14.3	i.p.
guinea-pig (male)	9.1	oral
guinea-pig (female)	8.1	oral
white leghorn	3.4	oral
turkey	5.9	oral
Japanese quail	16.5	oral
rainbow trout	4.7	i.p.
pig (female)	6 (total dose) ¹	oral

[WHO, 1990]

¹Both pigs receiving 2 mg/Kg daily were moribund and killed within 3 days, and both pigs receiving 1 mg/Kg daily were moribund and killed with 6 days [Szczech et al, 1973].

also observed in renal slices from rats fed with OTA 2mg/Kg bw for 2 days [Storen et al, 1982]. Renal phosphoenolpyruvate carboxykinase activity (PEPCK) was selectively lowered by 50%. This may be an early sign of OTA-renal damage.

Suzuki et al. [1975] observed a 60% decrease in hepatic glycogen levels and a concomitant increase in serum glucose and blood and hepatic lactate levels in rats after daily administration of OTA 5 mg/Kg for 3 days. A single dose of 15 mg/Kg bw decreased the activity of glycogen synthetase by 46% and increased the activity of phosphorylase by 40%. In vitro studies showed that OTA inhibited hepatic mitochondrial respiration primarily by altering the membrane permeability [Moore & Truelove, 1974].

Addition of OTA to renal cortical slices resulted in an increase in ⁴⁵Ca accumulation [Berndt et al, 1984]. Altered calcium homeostasis has been suggested as an event which might mediate cellular injury and/or death [Schanne et al, 1979]. It has been reported previously in our lab that in hepatic microsomes OTA strongly enhanced lipid peroxidation, and this led to an inhibition of calcium uptake [Khan et al, 1989; Rahimtula et al, 1988; Omar et al 1990].

OTA is a potent inhibitor of protein synthesis both in bacteria and mammalian cells. Studies have revealed that OTA competitively inhibits phenylalanyl-tRNA synthetase activity, which was proposed to be due to the phenylalanine moiety on

OTA [Creppy et al, 1984].

Carcinogenicity of OTA has been demonstrated in mice and rats, though OTA did not produce genetic or related effects in a variety of short term tests [Umeda et al, 1977; Wehner et al, 1978]. Long-term low dose feeding experiments, demonstrated that OTA is a hepatic and renal carcinogen in mice [Bendele et al, 1985; Kanizawa & Suzuki, 1978; Kanizawa, 1984]. It has been reported that administration of OTA to male and female rats for up to 2 years caused uncommon tubular cellular adenomas and carcinomas of the kidney [Boorman 1988]. OTA has also been shown to cause single strand breaks in DNA isolated from livers and kidneys of rats that had been fed the equivalent of 4ppm OTA for 12 weeks [Kane et al, 1986a&b]. OTA, when tested at 20 and 100 μ g/plate, did not have any effect in a Bacillus subtilis Rec-assay which measures DNA damage [Ueno & Kubota, 1976]. Assays using a number of Salmonella typhimurium strains have not demonstrated bacterial mutagenicity with dose of OTA up to 1000 μ g/plate, with or without exogenous metabolic activation [Würgler et al, 1991; Kuczuk et al, 1978; Wehner et al, 1978]. Human exposure, as demonstrated by the occurrence of OTA in food, blood, and in human milk, has been observed in various countries in Europe [Gareis et al, 1988]. Available epidemiological information indicates that Balkan nephropathy may be associated with the consumption of foodstuffs contaminated by this toxin. A

highly significant relationship has been observed between Balkan nephropathy and tumours of the urinary tract, particularly with tumours of the renal pelvis and ureters. However, no data have been published that establish a direct causal role of OTA in the etiology of such tumours.

1.1.3 Absorption and metabolism

In a study on rats exposed by gavage to a single dose of OTA at 10 mg/Kg bw, Galtier [1974b] found the highest tissue level of unmetabolized OTA in stomach wall during the first 4 hr following administration. The small and large intestine and caecum contained small amounts of unchanged OTA, and it was concluded that OTA was absorbed mainly from the stomach. Small amounts of OTA (1-3% of the total dose) were detected in the caecum and the large intestine, in the form of the isocoumarin moiety (OTA α), most likely as the result of the hydrolysing action of the intestinal microflora [Galtier & Alvinerie, 1976; Hult et al, 1976]. In a study on intestinal absorption using the same animal species, Kumagai & Aibara [1982] came to the conclusion that the site of maximal absorption of OTA was the proximal jejunum. Lee and co-workers [1984] studied the absorption and tissue distribution in Swiss mice over a 48-h period, after the administration of a single dose of 25 mg OTA/Kg body weight. The results

suggested that absorption mainly takes place in the duodenum and the jejunum. Recently, data from our lab has shown that the OTA was absorbed mainly from the duodenum and the jejunum, although some absorption did occur in the stomach [Barrowman et al, 1993]. Absorption is largely dependent on the absorbing area of the organ. Since the small intestine has a larger surface area than the stomach, presumably more absorption would occur in the small intestine than in the stomach.

In experimental studies on swine ingesting feed containing OTA, residues of this toxin were found in all tissues investigated. In decreasing order of concentration, these were kidney, liver, muscle, adipose tissue [Krogh et al, 1974]. A subsequent study revealed that the concentration of OTA residues in the blood of the pig was higher than those in the other tissues mentioned above [Mortensen et al, 1983]. When rats were exposed orally to an OTA dose of 10 mg/Kg bw, Galtier [1974a] recovered 0.3% of the administered dose in the whole kidneys, 0.9% in the whole liver and 0.6% in the total muscle tissue, 96 hr after exposure. In another experiment, using a single intraperitoneal injection of 1 mg OTA (labelled with ^{14}C in phenylalanine) per rat, Chang and Chu [1977] found that the kidney contained twice as much unchanged OTA as the liver after 0.5 hr, amounting to 4-5% of the total dose. In the study by Lee et al (1984), the largest amounts

of OTA found in the kidney (as indicated by staining intensity) were in the epithelium of the proximal convoluted tubules, and to a lesser extent in the distal convoluted tubules, the descending loop of Henle, and glomeruli and Bowman's capsule. Using pig renal cortical slices [Friis et al, 1988], or canine renal brush border and basolateral membrane vesicles [Sokol et al, 1988], it was found that OTA enters the kidney via the renal organic anion transport system.

OTA has been shown to bind to serum albumin by both in vivo and in vitro studies [Chang & Chu, 1977; Chu, 1971 & 1974b; Galtier, 1974a]. OT α has been detected in the urine and faeces of rats injected intraperitoneally with OTA [Nel & Purchase, 1968; Chang & Chu, 1977], indicating the cleavage of OTA to OT α and phenylalanine, under these conditions. Many environmental carcinogens and toxins require oxidative metabolism, most often by the cytochrome P-450 dependent monooxygenase system, in order to exert their toxic or carcinogenic effects [Miller & Miller, 1981]. In both albino and brown rats given OTA orally or intraperitoneally, 1-1.5% of the dose was excreted as (4R)-4-hydroxyochratoxin A and 25-27% as OT α in the urine [Storen et al, 1982]. In in vitro studies using hepatic microsomes from the pig, rat, and man, both (4R)- and (4S)-4-hydroxyochratoxin A were produced in a hydroxylation process involving cytochrome P-450 [Stormer &

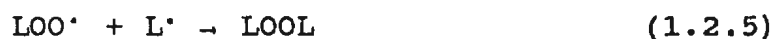
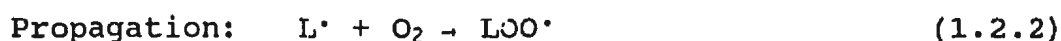
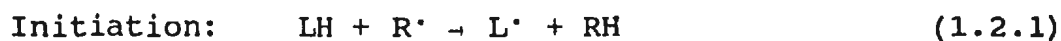
Pedersen, 1980; Stormer et al, 1981]. OTA is known to be metabolized by rabbit hepatic microsomes to an additional metabolite, 10-hydroxy-OTA [Ritchie & Idle, 1982]. 4-Hydroxyochratoxin A is non-toxic to rats in amounts up to 40mg/Kg body weight [Hutchison et al, 1971]; thus, it has been concluded that microsomal hydroxylation to this metabolite most likely represents a detoxification reaction.

As mentioned above, OTA has been detected in the urine and some faecal samples of bacon swine and rats. OTA has also been shown to be present in the milk of rabbits and goats. In the former East Germany, a study of human milk obtained from women in two hospitals revealed that 11.1% contained OTA [Bauer & Gareis, 1987; Gareis et al, 1988].

1.2 Lipid peroxidation

1.2.1 Lipid peroxidation

Membranes in eukaryotes contain many essential polyunsaturated fatty acids (PUFA). A free radical (R^\cdot) which has sufficient energy to abstract a hydrogen atom from a methylene carbon of a membrane unsaturated fatty acid (LH) can initiate a chain reaction in bulk lipid. This reaction sequence is known as **lipid peroxidation** [Gutteridge, 1988]. Once the free-radical chain reaction is initiated, it propagates until the unsaturated lipid is exhausted or two free radicals destroy each other to terminate the chain. Lipid peroxidation reactions can be classified into three main stages, these are: initiation, propagation, and termination.



where: R^\cdot = a free radical; LH = lipid

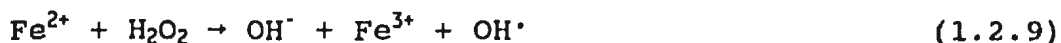
L^\cdot = lipid radical; LOO^\cdot = lipid peroxy radical.

If radical chain reactions are not terminated by the reaction of two radicals, they proceed concurrently, further destroying membrane lipid phase, especially lipid membrane.

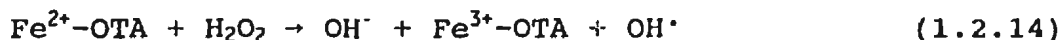
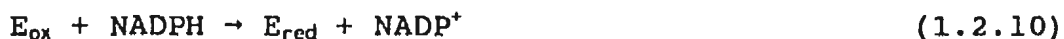
In general, superoxide radicals and transition metal ions such as iron ions play a central role in the initiation of lipid peroxidation in biological systems. Ferrous ions are themselves free radicals and they can take part in electron transfer reactions with molecular oxygen:



Generation of $\text{O}_2^{\bullet -}$ by any source in the presence of ferrous ions can lead to the formation of hydroxyl radicals ($\bullet\text{OH}$):



It was previously shown [Omar et al, 1990; Hasinoff et al, 1990] that in a reconstituted system consisting of NADPH, the flavoprotein NADPH-cytochrome P-450 reductase and Fe^{2+} ions, OTA stimulates superoxide and hydroxyl radical formation in liver by the following reactions:



in which E_{ox} and E_{red} are the oxidized and reduced forms respectively of NADPH-cytochrome P-450 reductase. The $\text{Fe}^{3+}\text{-OTA}$ complex is reduced very rapidly to $\text{Fe}^{2+}\text{-OTA}$ which, after binding to molecular oxygen, is transformed to a reactive species that initiates lipid peroxidation.

The products of lipid peroxidation include lipid epoxy alcohols, epoxides, hydroperoxides and short chain compounds including malondialdehyde (MDA), ethane, pentane, and 4-hydroxy alkenals [Gutteridge, 1987; Kappus, 1985] (Fig. 2 & 3). Malondialdehyde is the main defined reaction product measurable in a biological system which undergoes lipid peroxidation [Dahle et al 1962]. MDA is commonly detected by the thiobarbituric acid (TBA) reaction, which is shown in Fig. 4. The MDA-TBA complex is pink in colour, and has a absorbence at around 535nm. It is the most widely used method because of its simplicity and sensitivity. Ethane is another product. Measurement of ethane exhalation can be used as an in vivo indicator of lipid peroxidation, but a limitation of this method is that it does not identify the organ responsible for ethane production. It has been shown that the rate of ethane exhalation by rats treated with OTA was considerably higher than the ethane exhaled by control rats [Rahimtula et al, 1988]. This indicated that OTA stimulates lipid peroxidation in vivo, but it was not clear which organ(s) caused this elevation of lipid peroxidation. In addition, oxygen uptake can be measured as an indicator of lipid peroxidation in the cell, since oxygen is consumed to produce peroxy radicals during lipid peroxidation. Subsequent decomposition reactions of lipid peroxides can also consume oxygen. However, it is not as widely used as the MDA-TBA reaction.

Fig. 2. Scheme representing the formation of ethane during lipid peroxidation induced by hydroxyl radicals [Kappus,1985]

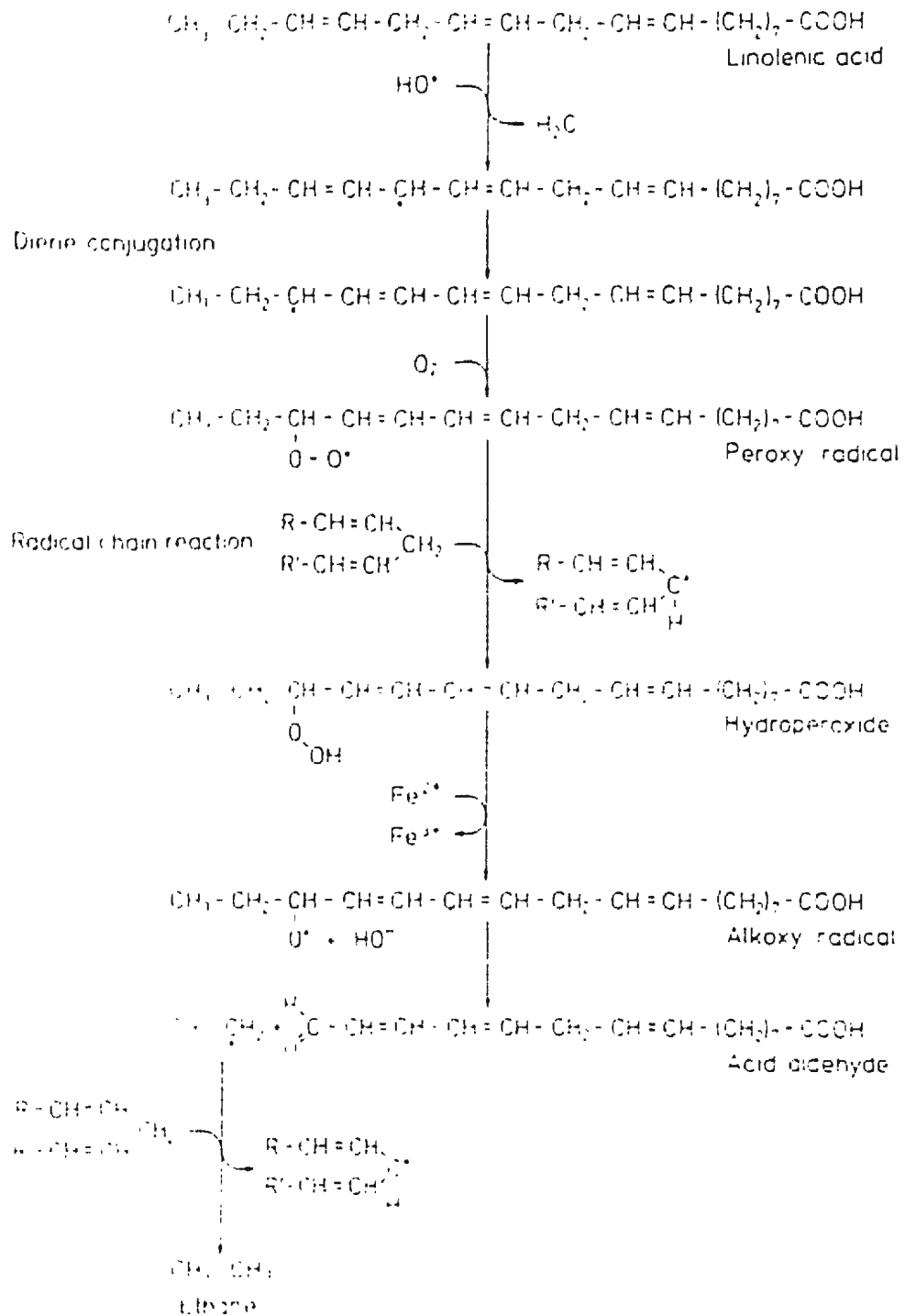


Fig. 3. Scheme representing the formation of malondialdehyde during lipid peroxidation induced by ferrous-oxygen complexes or by hydroxyl radicals [Kappus, 1985]

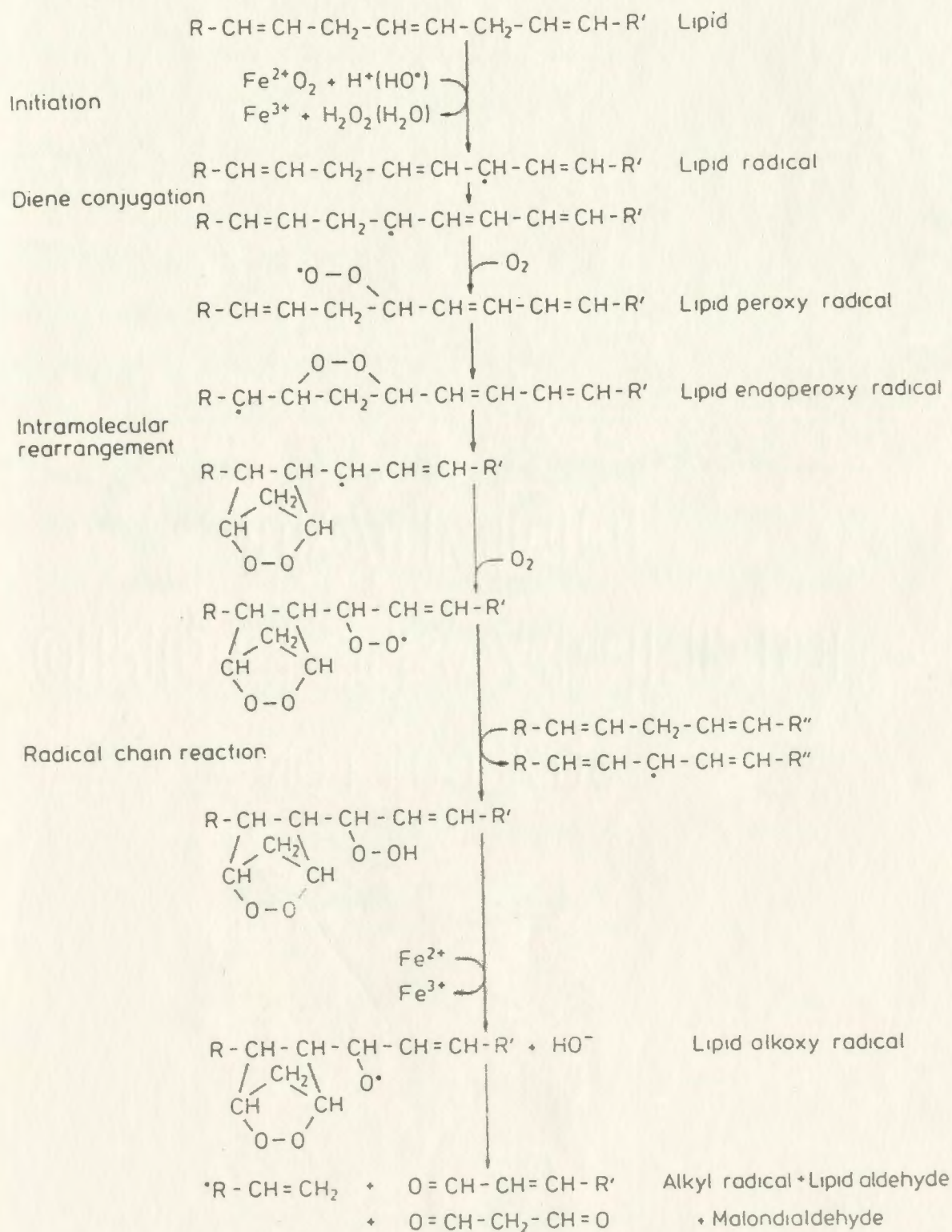
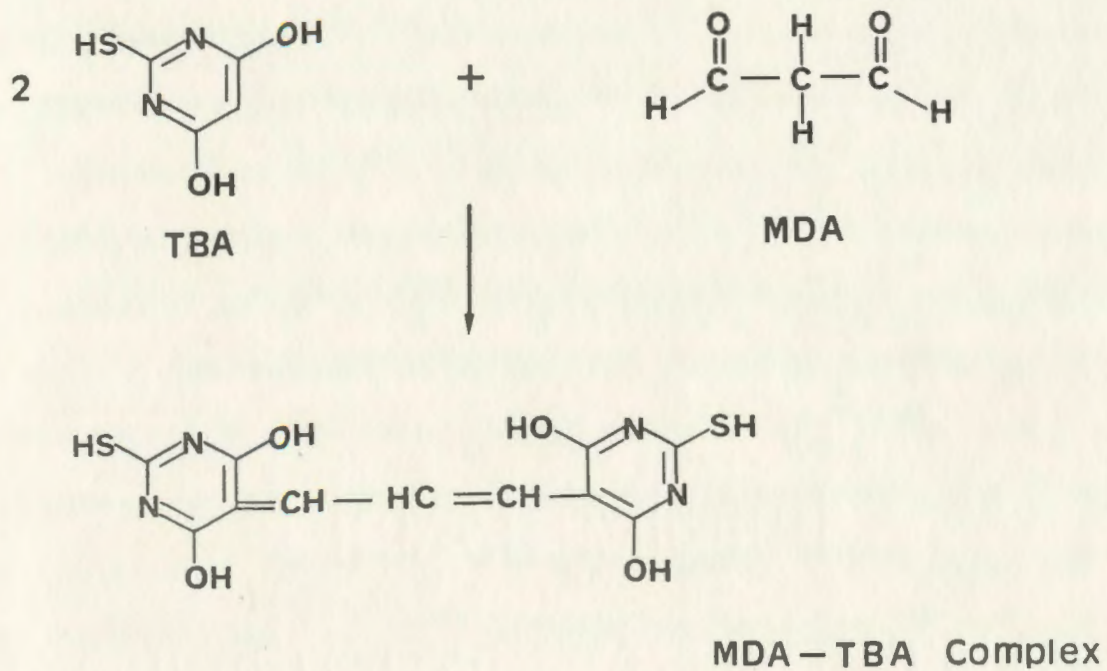
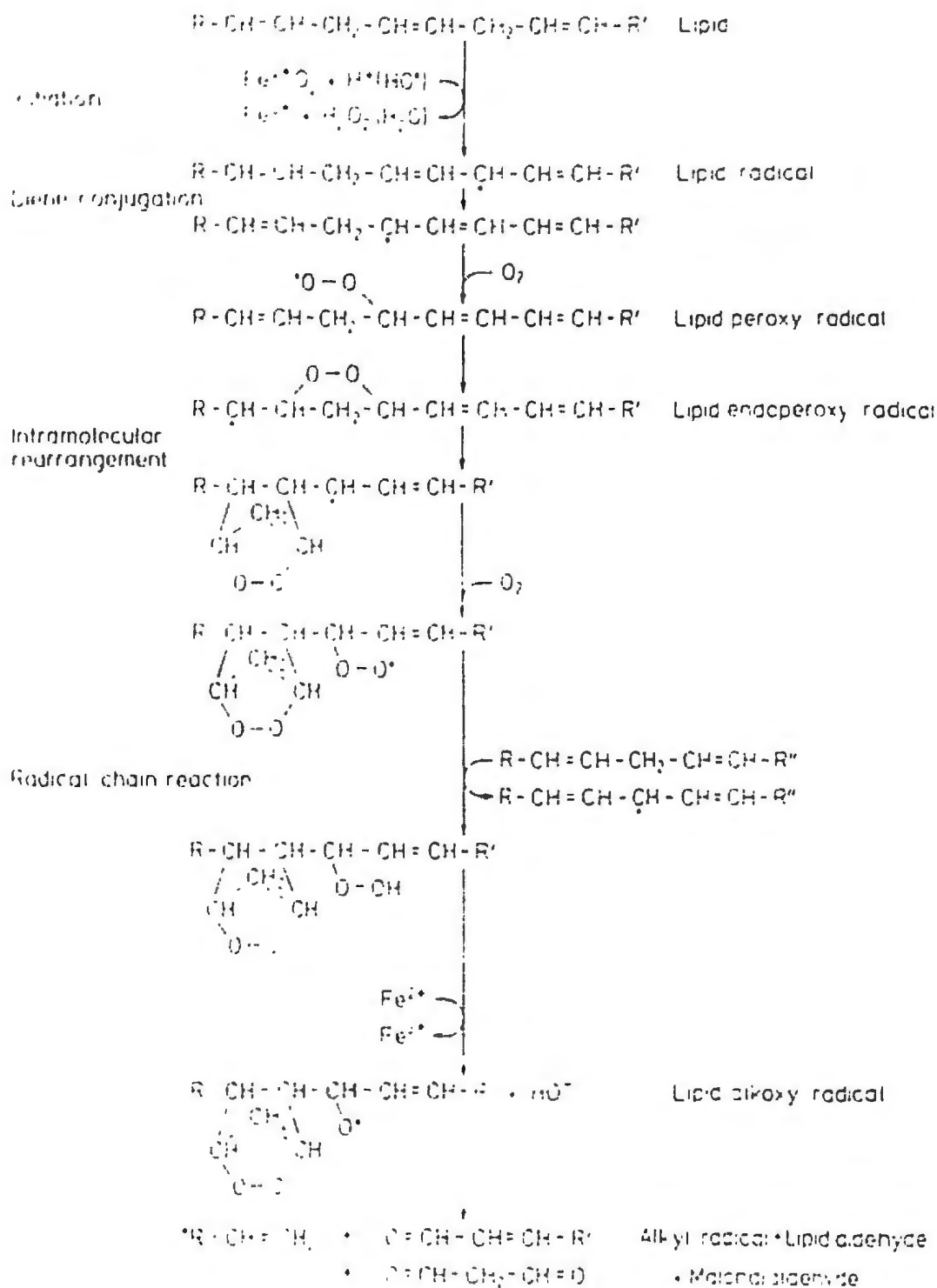


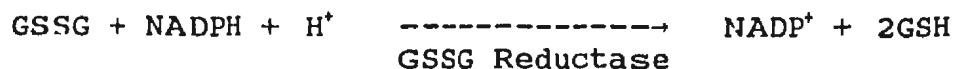
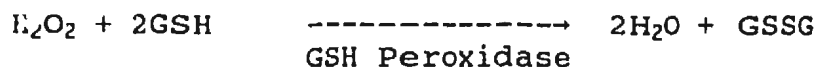
Fig. 4. Chemical reaction of MDA-TBA formation





1.2.2 Biological defence systems against lipid peroxidation

The potential for generating oxygen radicals is great in biological systems. Thus, a number of defence systems exist to protect against oxygen radicals. These include many kinds of antioxidants and radical scavengers. The advent of oxidative stress in vivo often leads to increased levels of antioxidant enzymes which serve to protect the organism/tissue from the deleterious effects of reactive oxygen species and lipid peroxidation products [Barros et al, 1991]. Superoxide dismutase (SOD) rapidly catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2 . Catalase, a haemoprotein, dismutates H_2O_2 to O_2 and water. H_2O_2 if left intact can react with Fe^{2+} to yield HO^{\cdot} radicals. Thus, the combination of SOD and catalase effectively eliminates the precursors of the very reactive and deleterious HO^{\cdot} radicals. Glutathione peroxidase, a Se containing enzyme, and glutathione reductase together with GSH (glutathione; γ -glutamylcysteinylglycine), are also involved in the removal of H_2O_2 :



Another enzyme, Glutathione S-transferase, is not directly involved in the removal of free radicals, but catalyses the

conjugation of an electrophilic xenobiotic with endogenous GSH. DT diaphorase catalyzes the oxidation of NADPH and NADH [Ernster, 1967].

Vitamin E (α -Tocopherol) is one of the most powerful antioxidants protecting membranes from peroxidative damage. The tocopherol moiety in Vitamin E reacts with lipid radicals, removes peroxy radical, thereby breaking the radical chain reaction [Packer et al, 1979].

1.2.3 Cellular toxicity

Lipid peroxidation has been implicated as a mechanism by which numerous organic chemicals produce toxicity. It is evident that lipid peroxidation reactions can destroy the biological membranes in which they occur. Plasma membranes of cells as well as intracellular membranes lose their ultrastructural architecture when cross-linking occurs between acyl chains, and long chain PUFA are depleted. These changes impair membrane fluidity and permeability [Bruch & Thayer, 1983; Vladimirov et al 1980].

Lipid peroxidation also inactivates many enzymes and protein receptors, among which the best known are the loss of cytochrome P-450 [Hogberg et al, 1973] in microsomes, and the inactivation of membrane Ca^{2+} -ATPase [Waller et al, 1983]. This inactivation can be caused simply by loss of membrane

structure: the enzyme or the receptor undergoes conformational changes which result in activation or inactivation of the respective protein. For example, the membrane Ca^{2+} -ATPase is inactivated because of oxidation of essential sulfhydryl groups in the enzyme [Jones et al, 1983], resulting in defective control of cytosolic calcium.

Lipid peroxidation consumes amounts of oxygen, which may lead to a critical condition in cells where the oxygen supply is limited [Kappus, 1985]. It also leads to depletion of protective mechanisms involving antioxidants, glutathione, etc.

In general, lipid peroxidation is a highly destructive process. Its specificity is low and it alters or destroys a great number of biomolecules. Cells, organs or whole organisms lose structural architecture and/or biochemical functions.

1.3 Calcium homeostasis

1.3.1 Introduction

The important role of calcium in cellular function has been well recognized during the last decade. The involvement of calcium is mainly through: 1) maintenance of intracellular calcium homeostasis, and 2) regulation of cellular functions through the calcium messenger systems.

The processes that regulate intracellular calcium are intricate, numerous, and diverse. Calcium homeostasis is maintained by a variety of transport systems in several different membranes: calcium gates, pumps, and channels operate to keep the free cytosolic calcium ion, $[Ca^{2+}]_i$, within a normal physiological range. Calcium homeostasis is also dependent upon the status of other cellular constituents, including sulfhydryls and glutathione [Johns et al, 1983]. The calcium messenger system is a highly organized system which contains the signal system, the calcium receptor system, calcium homeostatic processes, and the calcium response system [Pounds, 1990]. It has a central role in mediating many different cellular functions, which are generally of three types: cellular movement, including muscle contraction, amoeboid movement, and cytoskeletal functions; information processing, such as nerve impulse conduction and sensory

mechanisms; and the actions of hormones, including growth factors, on cellular growth and differentiation. Based upon the central role of the calcium messenger system in these aspects of cellular function, it is logical, if not inevitable, to examine possible disturbances in calcium homeostasis as underlying mechanisms of toxicant action.

In addition, proximal cells are the major site of calcium reabsorption [Hook & Goldstein, 1992], and as mentioned before, the proximal tubule is the primary target of OTA. Thus, a disturbance in calcium homeostasis in the proximal tubule by OTA could play a role in its nephrotoxic effect.

The extracellular calcium concentration is about 0.5 - 1.3 mM, and the intracellular free calcium concentration is close to 0.1 μ M, depending on the cell type [Dawson, 1990]. Maintenance of the concentration difference depends on two features of the plasma membrane: its low permeability to calcium and the presence of membrane-bound ATP-dependent pumps and $\text{Na}^+/\text{Ca}^{2+}$ exchangers that drive calcium out of the cell against the concentration gradient [Carafoli, 1987]. It has become increasingly clear in the last decade that this transport mechanism is tightly regulated, and that this regulation can affect the cytoplasmic $[\text{Ca}^{2+}]$. In addition, there are important internal, uptake-backflux systems associated with the sarcoplasmic reticulum (SR) in muscle cells, the endoplasmic reticulum (ER) in non-muscle cells, the

mitochondrial inner membrane, secretory vesicles, and the membranes of other organelles. Among them, the ER was established as a major calcium storage site in non-muscle cells [Somlyo et al, 1985]. Mitochondria are now generally considered not to play a major role in the cytosolic homeostasis of calcium [Carafoli 1987].

1.3.2 Endoplasmic reticular calcium transport

The ER in non-muscle cells takes up calcium from the cytosol and releases it back, thereby contributing to the regulation of cytosolic calcium levels. Of the two processes, uptake and release, the former is much better understood. Though several pathways for the release of calcium have been described [Kraus-Friedmann, 1990], the exact physiological mechanism(s) responsible for the release of calcium are not fully understood.

1.3.2.1 Calcium uptake

The uptake of calcium by the ER is due to the activity of an ATP-dependent calcium pump. The pump is a high-affinity calcium ATPase, which interacts with calcium; but transports calcium with a low total capacity. The calcium ATPase is an E1-E2 transport ATPase [Carafoli, 1987], which is postulated

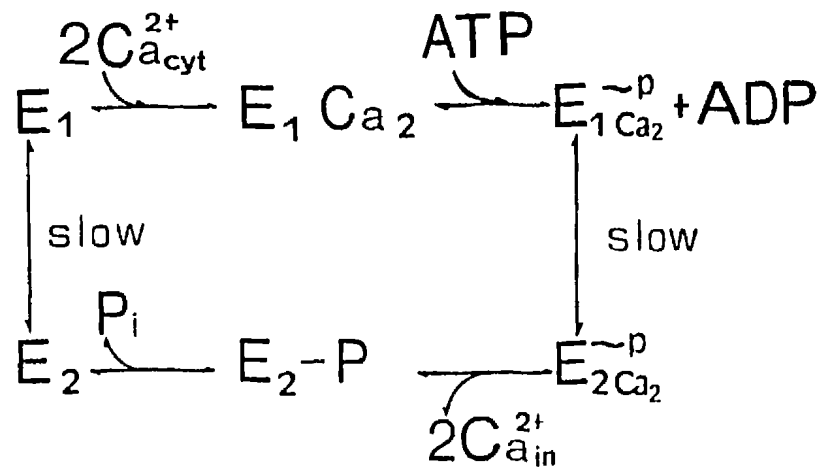
to exist in two different conformational states E1 and E2 at different stages of the reaction mechanism, and to conserve the ATP energy intramolecularly in the form of an acyl phosphate, most likely an aspartyl phosphate (Fig. 5). The phosphoprotein intermediate in pig renal microsomes has a molecular mass of about 115 kDa [Parys et al, 1985], but this may vary in different cell types.

Calcium uptake is Mg^{2+} -dependent, because MgATP is the substrate of the enzymatic reaction [Kraus-Friedmann, 1990]. Oxalate is the anion generally used to increase calcium uptake into the ER, as a result of its permeation into the lumen and the formation of an insoluble calcium complex [Parys et al, 1985, Heilmann et al, 1984].

Microsomal calcium pump activity has been shown to be very sensitive to oxidative damage and lipid peroxidation [Waller et al, 1983], which may be due to the oxidation of essential sulfhydryl groups in the enzyme [Jones et al, 1983].

NADPH inhibits the ATP-dependent calcium pump of hepatic microsomes from rats [Prasad et al, 1986]. It was proposed that the inhibition of calcium pump activity by NADPH is mediated by the direct oxidation of the critical pump protein thiol groups by the ferric-cytochrome P-450-oxygen pathway since GSH was found to reverse the inhibitory effect of NADPH [Holtzman et al, 1989, Srivastava, 1990]. These authors have suggested that activity of the microsomal calcium pump in vivo

Fig. 5. Equation of Ca-ATPase E1-E2 model [Carafoli, 1987]



is maintained by the cytochrome P-450 system which tends to oxidize sulfhydryls and thereby decrease activity: the enzyme thiol:protein disulfide oxidoreductase (TPDO) reduces these protein disulfides to reactivate the pump. The authors proposed that a specific isozyme(s) of cytochrome P-450 is responsible for the inhibition of the hepatic microsomal calcium pump and that a substrate or inhibitor for that particular isozyme would protect against NADPH inhibition of the calcium pump. Srivastava et al [1990] concluded that a few cytochrome P-450 isozymes, most likely members of the CYP11B and CYP11A family, could be involved in the inhibitory effect.

1.3.2.2 Calcium backflux

In contrast to the process of calcium uptake, which is the function of a single activity, that of the calcium-ATPase, the release of calcium from ER can occur in a multitude of ways. Of these, the release of calcium by the reversal of the calcium-ATPase is the best understood. This reaction was studied in the liver by Webb and Anders [1985] using calcium-loaded microsomal vesicles. This preparation accumulated calcium in the presence of ATP to a maximal value. Upon the dilution of the calcium-loaded microsomal vesicles into a backflux medium, calcium release occurred, the rate of which

more than doubled upon the addition of ADP and P_i . When studied under appropriate experimental conditions, the release of calcium was associated with the synthesis of ATP.

Other ways by which calcium release can be evoked is by the addition of GTP or IP_3 to calcium-loaded microsomal vesicles [Dawson et al, 1987]. Calcium release can also be caused by direct inhibition of the calcium-ATPase by GTP [Kimura et al, 1990]. The most effective way of eliciting calcium release from the microsomal fraction so far identified is by the oxidation of critical -SH groups [Zhang et al, 1990]. This mechanism is likely to be involved in the rise of cytosolic calcium observed in many instances of hepatocellular injury.

1.3.3 The role of calcium ions in the mechanism of toxic insults

Because of the complexity and diversity of calcium homeostatic processes and calcium-mediated responses, it is useful to consider the many ways in which a toxicant may act on the calcium messenger system. Generally, calcium-related toxicity can be shown as a direct alteration of calcium homeostasis, and/or deregulation of calcium-mediated cellular functions.

Toxicants may directly alter calcium homeostasis by

substituting for calcium at specific sites of calcium mobilization, transport, or storage; or by acting on specific cellular and molecular constituents of Ca^{2+} -homeostatic processes, such as calcium pumps or gates, which may occur at molecular sites that are independent of, or remote from calcium, not at the calcium transport or binding site. Other divalent metals are commonly involved in substitution mechanisms, and the latter type of interaction is observed with many organic toxicants. Toxicants that dissipate the Na^+ gradient will secondarily alter calcium homeostasis in cells where the $\text{Ca}^{2+}/\text{Na}^+$ exchange is a component of calcium homeostasis. Na^+ transport is affected by numerous drugs, thiol status, and energy metabolism. Toxicants may secondarily alter calcium homeostasis through non-specific effects on cellular function that are biochemically and functionally remote from the processes of calcium transport and storage, such as altered calcium permeability and transport properties of the membrane [Michaelis, 1983].

The acute toxicity of organic toxicants has been linked to altered calcium homeostasis [Schanne et al, 1979; Moore, 1982; Orrenius et al, 1989]. The exposure of hepatocytes to CCl_4 and certain other halocarbons, carbon disulfide, or thioacetamide results in rapid loss of Ca^{2+} -ATPase activity and loss of the ability of the endoplasmic reticulum to sequester calcium [Moore et al, 1976; Lowrey et al, 1981; Long & Moore,

1986; Tsokos-Kuhn, 1989]. Other toxicants such as cisplatin may increase renal Ca^{2+} -ATPase activity [De Witt et al, 1988].

Maintenance of normal $[\text{Ca}^{2+}]$ homeostasis is also dependent upon the status of other cellular constituents, including sulfhydryl status and GSH [Johns et al, 1983]. Agents that cause oxidative stress, or which deplete cellular glutathione through conjugation reactions will perturb calcium fluxes and intracellular compartmentation resulting in deregulation of cellular function and cell death [Johns et al, 1983; Orrenius et al, 1984].

In our lab, it has previously been shown that OTA administration to rats increased ethane exhalation (an index of in vivo lipid peroxidation) [Rahimtula et al, 1988] and inhibited the hepatic microsomal calcium pump activity [Khan et al, 1989]. Also, OTA addition to hepatic microsomes in the presence of NADPH greatly enhanced lipid peroxidation and strongly depressed microsomal calcium uptake. Moreover, a good correlation was observed between inhibition of ATP-dependent calcium uptake and enhanced lipid peroxidation as judged by MDA formation [Khan et al, 1989]. However, the effects of OTA on calcium homeostasis need to be examined in great detail.

1.4 Objective of the thesis

The objective of my research is to investigate the effect of in vivo OTA administration to rats on renal cortical (i) microsomal calcium pump activity, (ii) lipid peroxidation and (iii) cytosolic antioxidant enzyme levels. The effect of in vitro addition of OTA to renal cortical microsomes on calcium uptake and lipid peroxidation is also examined. The purpose of these experiments is to establish the importance (if any) of lipid peroxidation in OTA-induced alteration in renal microsomal calcium uptake.

These studies are considered relevant in view of the fact that OTA is primarily a nephrotoxin and previous studies have shown that (i) administration of OTA to rats resulted in enhanced lipid peroxidation in vivo as evidenced by increased ethane exhalation [Rahimtula et al, 1989], (ii) OTA stimulated NADPH-dependent lipid peroxidation in hepatic microsomes in vitro [Rahimtula et al, 1989; Omar et al, 1990], and (iii) OTA inhibited hepatic microsomal calcium uptake both in vivo and in vitro [Khan et al, 1989].

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

ATP, butylated hydroxytoluene (BHT), 1-butanol, dimethyl sulfoxide (DMSO), ethylenediamine tetracetic acid-disodium salt (EDTA), NADPH, ochratoxin A (OTA), 2-thiobarbituric acid (TBA), xanthine, 2,5-dichlorophenolindophenol (DCPIP), Tween-20, 1-chloro-2,4-dinitrobenzene (CDNB) (recrystallized from ethanol), dicoumarol, reduced glutathione (GSH), oxidized glutathione (GSSG), ferricytochrome c, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $^{45}\text{CaCl}_2$ (specific activity 16.1 mCi/mg) was purchased from Du Pont Canada Inc. (Montreal, Que., Canada), and [γ - ^{32}P] ATP (specific activity 5,000 Ci/mmol) was purchased from Amersham Canada Limited (Oakville, Ont., Canada). All other chemicals were of the highest grade commercially available.

2.2 Methods

2.2.1 Animal treatment

Male Sprague-Dawley rats (225 - 300 g) were obtained from Charles River Canada, La Prairie, Quebec and maintained on standard laboratory rat chow and water ad lib. OTA was administered intraperitoneally (i.p.) or orally (p.o.) as a 2 mg/mL solution in 0.1 M sodium bicarbonate. The various treatments are listed below:

- 1) OTA (0.5, 1, 2, or 4 mg/Kg bw), i.p. daily for four days,
- 2) OTA (0.5, 1, or 2 mg/Kg bw), i.p. daily for eight days,
- 3) OTA (5 mg/Kg bw) as a single dose,
- 4) OTA (10 mg/Kg bw), i.p. as a single dose,
- 5) OTA (10 mg/Kg bw), p.o. as a single dose.

Control rats received an equivalent amount of vehicle (0.1 M sodium bicarbonate). Each animal was anaesthetized with ether and the blood was taken from the abdominal aorta at the indicated times after treatment, then a cut was made up through the diaphragm and both kidneys were excised and weighed. Renal cortical microsomes were prepared as described in 2.2.2.

2.2.2 Preparation of microsomes

Rat renal cortical microsomes were isolated from untreated or OTA-pretreated rats. All the following procedures were performed at 0 - 4°C according to the method described by Rahimtula et al [1979]. The kidneys from each

rat were excised and pooled separately (unless specially indicated) in ice-cold 0.1 M potassium phosphate buffer (pH 7.4). The kidneys were then sliced in half and the cortex was carefully cut off from the medulla with scissors. The renal cortex was minced into fine pieces and homogenized in 3 volumes (w/v) of 0.1 M potassium phosphate buffer (pH 7.4) by a motor-driven Potter-Elvehjem homogenizer. In some cases, 2 mL of the homogenate was taken, mixed with EDTA (final concentration 1 mM), and stored for lipid peroxide measurement using MDA-TBA method. The rest of the homogenate was centrifuged at 10,000 x g for 15 min.

Microsomes were isolated from the 10,000 x g supernatant by centrifugation at 105,000 x g for 75 min. In some cases, the 105,000 x g supernatants (cytosol) were stored at -70°C for up to one week before used for measurement of enzyme activities. The microsomal pellets were resuspended by homogenization in 0.1 M phosphate buffer (pH 7.4) in a volume equal to the initial weight of the renal cortex (to yield a protein concentration of about 10 mg/mL).

For MDA measurement, EDTA (final concentration 1 mM) was added to 1.5 mL of the microsomal suspensions which were stored separately. All samples were stored at -70°C for up to one week before used. Protein concentration was determined by the method of Lowry et al [1951], using bovine serum albumin as a standard.

2.2.3 In vitro preincubations

Prior to measuring calcium uptake, cortical microsomes (1.0 mg protein/mL) from control rats were preincubated with NADPH (0.75 mM) in the presence or absence of OTA. For OTA concentration studies, renal cortical microsomes (1.0 mg protein/mL) were preincubated in 0.1 M potassium phosphate buffer (pH 7.4) for 10 min at 37°C with a range of OTA concentrations (0 to 1.0 mM; added in a maximum of 5 μ L of DMSO/mL incubation volume) in the presence or in the absence of NADPH (0.75 mM).

For the time course, renal cortical microsomes (1.0 mg protein/mL) were incubated as above with OTA (1 mM) but for various time periods (2 min to 1 hr). At the end of the incubation, the mixtures were immediately cooled on ice and centrifuged at 105,000 x g for 30 min at 4°C to recover the microsomes. The supernatants were discarded and the microsomal pellets obtained were resuspended in imidazole-histidine buffer (30 mM imidazole, 30 mM histidine, 100 mM KCl; adjusted to pH 6.8 with imidazole) to a concentration of 0.5 mg protein/mL and assayed for calcium uptake as described below in 2.2.4.

2.2.4 Microsomal calcium transport

2.2.4.1 Measurement of calcium uptake

Calcium uptake activity by renal cortical microsomes from control or OTA-administered rats or in in vitro incubated microsomes was measured by the method of Moore et al [1976]. Microsomes (0.1 to 0.15 mg protein/mL) were added to the ice-cold imidazole-histidine buffer containing 5 mM MgCl_2 , 5 mM ATP, 5 mM ammonium oxalate, 20 μM CaCl_2 and 0.1 $\mu\text{Ci/mL}$ $^{45}\text{CaCl}_2$. A 0.5 mL aliquot was filtered immediately with the aid of a vacuum apparatus on pre-wetted glass microfibre filters (Whatman, type 934-AH; diameter 24 mm; pore size 0.2 μm) as zero time. The reactions were then started by placing the tubes in a 37°C shaking-water bath and at indicated time intervals, 0.5 mL aliquots were withdrawn and filtered in the same way as described above. After rapid washing with 10 mL of ice-cold buffer, individual filters were placed in separate scintillation vials and air dried. Bound radioactivity was monitored in a Beckman LS-1801 liquid scintillation counter after addition of 5 mL scintillation fluid (ScintiVerse II*, Fisher Chemical Co.).

For studies on the direct effect of OTA on cortical microsomes, OTA (0 to 1.0 mM) was added to the system at the indicated time points.

To monitor the effects of calcium concentration on the calcium uptake activity, microsomes (0.1 to 0.15 mg

protein/mL) were added to the same system described above but containing different amounts of CaCl_2 (0.5 to 100 μM) instead of the standard 20 μM CaCl_2 . The specified concentrations of Ca^{2+} refer to amounts of this metal added to the incubations and does not take into account the already existing Ca^{2+} in the membrane.

To show that the microsomal calcium uptake under these conditions is ATP-dependent, i.e. is due to a calcium ATPase, ATP was omitted from the system in specified experiments.

2.2.4.2 Determination of [ATP] during calcium uptake

Incubation Microsomes were preincubated with NADPH in the presence or absence of OTA (1 mM) for 10 min, centrifuged and the resuspended microsomes were incubated for calcium uptake as described in 2.2.4.1, except that CaCl_2 was used instead of $^{45}\text{CaCl}_2$. At timed intervals, 0.5 mL aliquots were withdrawn and mixed immediately with 2 mL of 0.6 M HClO_4 to stop the reaction. The samples were brought to a pH of ~6.5 by addition of 4 mL of 0.1 M potassium phosphate (pH 7.5) and 0.3 mL 30% KOH. The precipitated KClO_4 was removed by filtering through a 0.45 μ nylon filter (Cameo II, MSI, Westboro, MA) and ATP levels were measured in the filtrate by HPLC as described below.

Chromatographic conditions HPLC conditions were as

described by Stocchi et al [1987] with some modification. The HPLC system used was a Perkin-Elmer Series 4 Solvent Delivery system, a Model ISS-100 Auto-Injector, a Model 3600 Data Station, and an LC detector. ATP/ADP analyses were performed on a 5 μ m Supelco LC-18 Reversed Phase analytical column (4.5 mm x 24 cm) protected with a LC-18 guard column (4.5 mm x 5 cm) (Bellefonte, PA). The injection volume was 20 μ L. The mobile phase used for the separation of nucleotides consisted of two buffers: 0.1 M potassium phosphate, pH 6.0, containing 8 mM tetrabutylammonium hydrogen sulfate (buffer A) and 0.1 M potassium phosphate, pH 6.0, containing 8 mM tetrabutylammonium hydrogen sulfate and 30% (v/v) of methanol (buffer B).

The analyses were performed at room temperature with a flow rate of 1.5 mL/min and detection at 254 nm. The chromatographic conditions used were as follows:

Time (min.)	% A
0-2.5	100
5.0	80
10	60
13-18	0
18.1-22	100

The system was calibrated by injecting standard ATP/ADP solutions of known concentration. Initially, a 4-level calibration run was performed, giving a linear response

(standard curve) for standard ATP/ADP.

2.2.4.3 Measurement of calcium backflux

Microsomes (3 mg protein/mL) from control rats were loaded passively by incubating for 2 hr at 0-4°C in 100 mM KCl, 5 mM MgCl₂, 20 mM HEPES, pH 7.5 with 1 mM ⁴⁵CaCl₂ (0.7 µCi) in a total volume of 2.0 mL. Efflux of ⁴⁵Ca²⁺ was measured as described by Tsokos-Kuhn et al [1989] and initiated by diluting 100 µL of this mixture (in triplicate) into 2.9 mL of ice-cold buffer containing 100 mM KCl, 5 mM MgCl₂, 20 mM HEPES, 2 mM EGTA, pH 7.5 with different concentration of OTA at 0-4°C. At timed intervals, 0.5 mL aliquots were taken, filtered and washed in the same way as described in 2.2.4.1.

2.2.5 Measurement of lipid peroxidation

Lipid peroxidation in the samples was monitored by measuring the level of the MDA as described by Uchiyama and Mihara [1978]. In a 10 mL test tube, 0.5 mL (about 5 mg protein) of homogenate or microsomes was mixed with 3 mL of 1% H₃PO₄ to keep the pH of the medium at about 2.0. One millilitre of 0.6% aqueous TBA was then added and the mixture was heated at 100°C for 45 min. After cooling, 4 mL of 1-

butanol was added and the mixture was shaken vigorously. The butanol phase was separated by centrifugation and its absorbance at 535 and 520 nm was measured. The difference in absorbance at these two wavelengths was taken to avoid interference due to the protein [Uchiyama & Mihara, 1978]. Tetramethoxypropane was used as an external standard to calculate the MDA-TBA value as MDA is released by the hydrolysis of tetramethoxypropane. The level of MDA-TBA is expressed as nmol of MDA per mg protein.

2.2.6 Phosphorylation of ER vesicles

Protein phosphorylation levels were measured by labelling microsomes with [γ -³²P]ATP followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as described by Heilmann [1984, 1985, 1989] with some modification. Phosphorylation was carried out at 0°C in a volume of 0.2 mL and contained 37 mM Hepes (pH 6.8), 0.1 M KCl, 50 μ M CaCl₂, 2.5 mM azide, 1.85-7nM [γ -³²P]ATP (specific radioactivity 5,000 Ci/mmol), and 30 μ g microsomal protein. The reaction was stopped after 1 min by addition of 0.2 mL of a solution containing 8% SDS (w/v), 10% 2-mercaptomethanol (v/v) and 188 mM Tris-HCl (pH 6.8). Samples were incubated for 15 min at 37°C and then mixed with 50 μ L of 100% sucrose. SDS-PAGE was carried out by the method of Laemmli [1970] as

described by Spamer et al [1987]. Proteins were stained with Coomassie Brilliant Blue R-250 to distinguish the bands. Destained gels were dried by heat under vacuum and exposed to Kodak X-Omat RO film. The protein band corresponding to the [^{32}P]phosphorylated protein ($M_r \sim 116,000$) was then cut, solubilized with 0.25 mL of 30% H_2O_2 at 50°C and bound radioactivity was quantified by liquid scintillation counting.

2.2.7 Measurement of enzyme activities

2.2.7.1 Catalase

Catalase activity of cytosol was estimated by measuring the decrease in absorbance of H_2O_2 at 240 nm (20°C). The assay was carried out in 3 mL of 50 mM potassium phosphate buffer (pH 7) containing 0.1 mg protein/mL cytosol and 0.02% H_2O_2 . The reaction was started by addition of H_2O_2 (mixed well with a plastic paddle) and absorbance was recorded for about 30 sec [Aebi, 1984]. The molar absorbency of H_2O_2 is $43.6 \text{ M}^{-1}\cdot\text{cm}^{-1}$.

2.2.7.2 Superoxide dismutase (SOD)

Enzyme activity was measured by a modification of the method described by McCord and Fridovich [1969]. The assay was performed in 3 mL of 50 mM potassium phosphate buffer (pH

7.8) containing 100 μM EDTA in a thermostated cuvette at 25°C. The reaction mixture contained 10 μM ferricytochrome c, 100 μM xanthine, and 0.015-0.02 units xanthine oxidase to produce a rate of reduction of ferricytochrome c at 550 nm of 0.025 absorbance unit per min. Addition of cytosol reduced the rate of cytochrome c reduction in proportion to its SOD content and the decreased rate of absorbance change was recorded. Under these defined conditions, the amount of SOD (cytosol) required to inhibit the rate of reduction of cytochrome c by 50% is defined as 1 unit of activity.

2.2.7.3 Glutathione peroxidase

Glutathione Peroxidase activity was measured as described by Lawrence and Burk [1976]. The reaction system contained in a total volume of 1 mL: 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3 , 0.2 mM NADPH, 1 E.U. GSSG-reductase, 1 mM GSH, and 0.25 mM H_2O_2 . All ingredients except enzyme source (cytosol) and peroxide were combined at the beginning of each day. Cytosol (0.1 mL) was added to 0.8 mL of the above mixture and allowed to incubate for 5 min at room temperature before initiation of the reaction by the addition of 0.1 mL of 2.5 mM H_2O_2 . The rate of decrease in absorbance at 340 nm was recorded for 5 min and glutathione peroxidase activity was calculated from the slope of these lines as μmol

NADPH oxidized per min. Blank reactions with enzyme source replaced by distilled water were subtracted from each assay.

2.2.7.4 Glutathione reductase

Glutathione reductase activity was assayed spectrophotometrically in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.6), 0.1 mM NADPH, 0.1% bovine serum albumin (BSA) and 3.3 mM GSSG in a final volume of 1 mL. The reaction was started by adding cytosol (1.0 mg protein), and was followed by recording the rate of reduction of NADPH at 340 nm [Flohe & Gunzler 1984]. The extinction coefficient of NADPH is $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

2.2.7.5 Glutathione S-transferase

Glutathione S-transferase activity was measured as described by Habig et al [1974] using 5-chloro-2,4-dinitrobenzene (CDNB) as acceptor substrate. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.5), 1 mM GSH, 1 mM CDNB and cytosol (0.1 mg protein) in a total volume of 3 mL. The reaction was started by adding GSH, and the absorbance was followed at 340 nm. The extinction coefficient of CDNB is $9.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

2.2.7.6 DT diaphorase

DT diaphorase was assayed spectrophotometrically by a modified method of Ernster [1967]. The reaction mixture contained 25 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 40 mM 2,6-dichlorophenolindophenol (DCPIP), 10 μ M dicoumarol, 0.07% BSA and 0.001% Tween-20 in a final volume of 3 mL. The reaction was started by the addition of 0.1 mg cytosolic protein, and DCPIP reduction was recorded at 600 nm (25°C). The extinction coefficient of DCPIP is $2.1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

2.2.8 Statistical Analyses

Data were analyzed by the Student's "t" test or ANOVA when the multiple comparisons were done. The Benferroni test as post test was used in ANOVA to correct the "p" value obtained in the "t" test. The level of significance was taken as $p < 0.05$ or $p_g < 0.05$ for the "t" test or the Benferroni test, respectively.

C H A P T E R 3

3. Results

3.1 Biological observations on OTA treated rats

It was observed that 4 or 8 days after the multiple low dose OTA treatment or 12 hr after a single high dose OTA (10 mg/Kg, i.p.) treatment, the treated rats had an accumulation of fluid in their abdominal cavity.

Rats in some groups were weighed both before and after the treatment. Fig. 6 shows that after 4 days of treatment, rats in the control group gained weight of about 42%, from 184.5 g to 262.5 g ($p=0.0003$); but the gain of weight in OTA (4 mg/Kg, i.p., daily) treated group was not significant (from 193.8 g to 206.0 g). Comparing the weight gained in the two groups (78 g in the control group and 12.2 g in the treated group), the difference was significant ($p=0.0005$). Since the control rats were much bigger than the treated ones, it was not a surprise that their liver weights (10.28 g) were significantly higher ($p=0.001$) than those in the treated groups (7.05 g). In Table 2, values of the percentage of liver weight over body weight are shown, and there is no difference between the control (3.91 g) and treated (3.43 g) groups of the 4-day treatment. The weight of the kidneys in the 4-day treatment groups showed no difference between the

control and treated groups (Fig. 6), but the percentage weight of kidneys over body weight (0.75% bw) in the treated group were significantly ($p=0.0091$) higher than those (0.64% bw) in the control group.

Fig. 7a shows that after 8 days treatment, the weight of the rats dropped by about 22% ($p=0.0059$) in the 2 mg/Kg OTA (i.p., daily) group (from 233.3 g to 181.3 g), and remained unchanged in the two lower dose groups, but increased by about 19% ($p=0.0325$) in the control group (from 232.7 g to 277.0 g). Comparing the weight gained/lost in the control and OTA treatment groups after the 8 days (Fig. 7a), the difference was significant ($p<0.05$). The liver weights were lighter ($p<0.05$, ANOVA test) in the treated groups (about 10 g) than in the control group (about 12 g), but there was no difference observed in kidneys weights (Fig. 7b). Values of the percentage of liver/kidneys weight over body weight did not show any difference between control and the two lower dose groups, but the values of the 2 mg/Kg dose group were significantly higher than those of the control group (Table 2). In the 2 mg/Kg dose group, the percentage of liver weight over body weight (5.86%) was significantly higher than that in the control group (4.76%), and the percentage of kidneys weight over body weight (1.1%) was significantly higher (0.82%) than that in the control group.

Fig. 6. Comparison of body, liver and kidneys weights between control rats and OTA treated rats after four days treatment.

Rats were treated with OTA (i.p., 4.0 mg/Kg) or vehicle (bicarbonate) daily for 4 days, and weighed before and after the treatment. The liver and both kidneys were wiped dry and weighed.

- * denotes a significant ($p=0.0003$) difference in weight
- ** denotes a significant ($p=0.001$) difference in weight
- *** denotes a significant ($p=0.0005$) difference in weights gained between the control and treated rats.

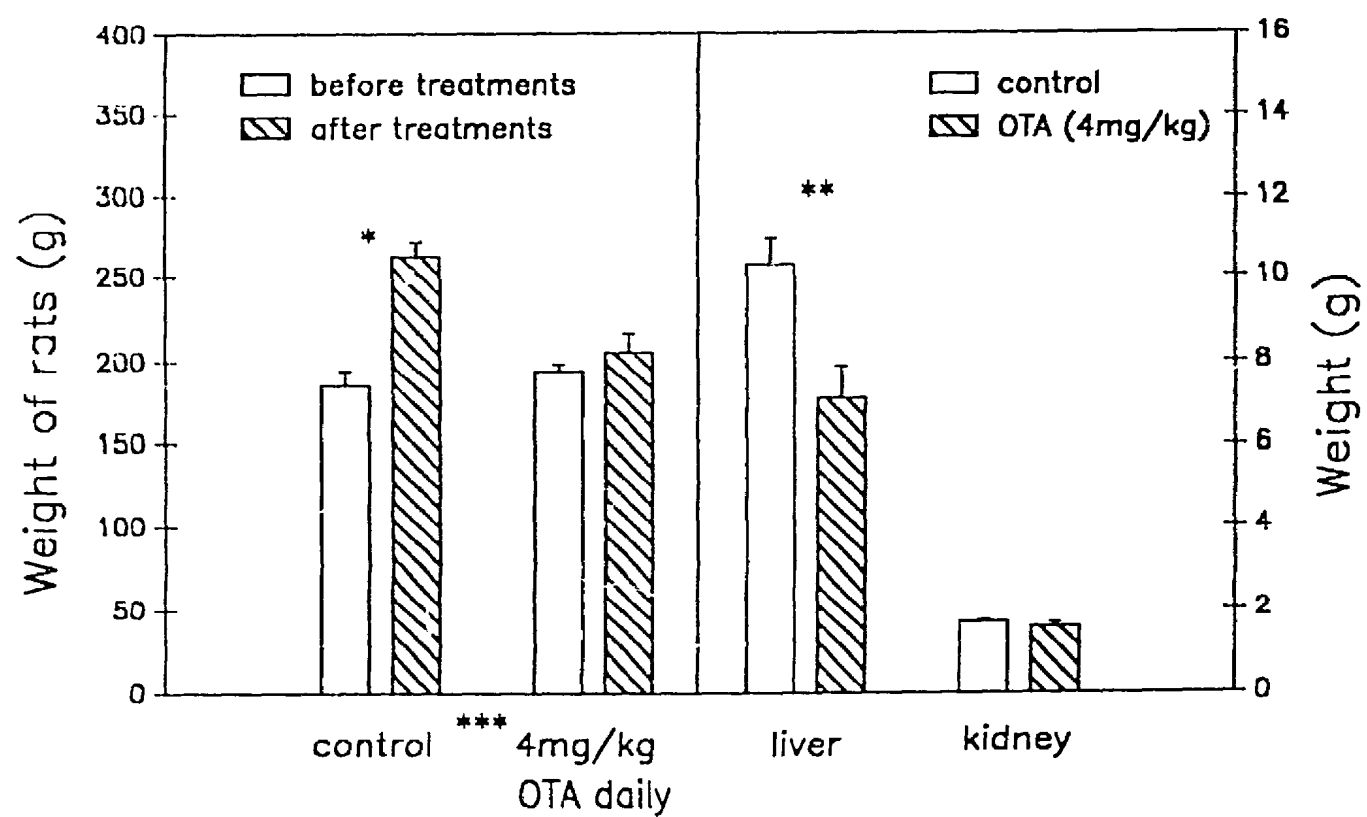


Fig. 7a. Changes in rat body weight after eight days of OTA treatment.

Fig. 7b. Comparison of liver and kidneys weights between control and OTA treated rats (8 days).

Rats were treated with OTA (i.p., 0.5, 1.0, 2.0 mg/Kg) or vehicle (bicarbonate) daily for 8 days, and weighed before and after the treatment. The liver and both kidneys were wiped dry and weighed.

* denotes a significant ($p=0.0325$, t test) difference in weight

** denotes a significant ($p=0.0095$, t test) difference in weight

*** denotes a significant ($p_0<0.05$, ANOVA test) difference in weight gained between the control and the treated rats.

**** denotes a significant ($p_0<0.05$, ANOVA test) difference in weight

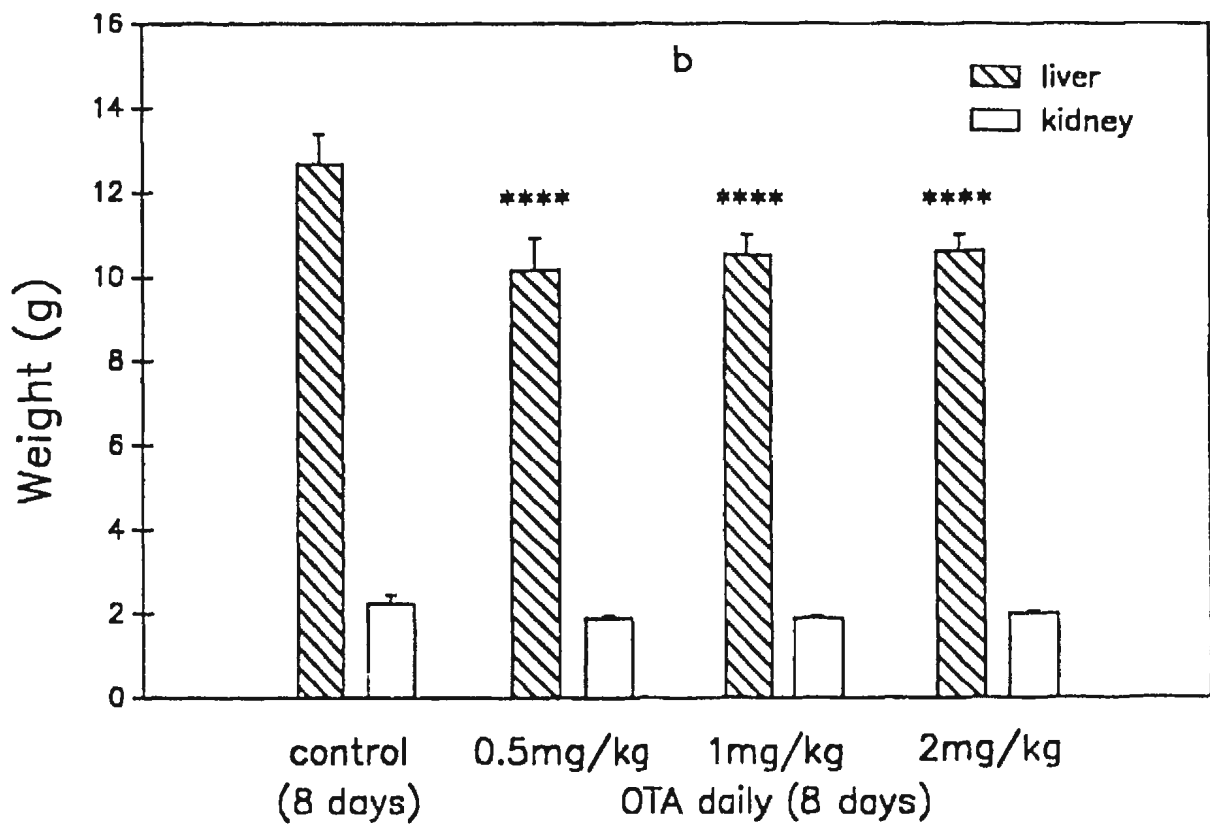
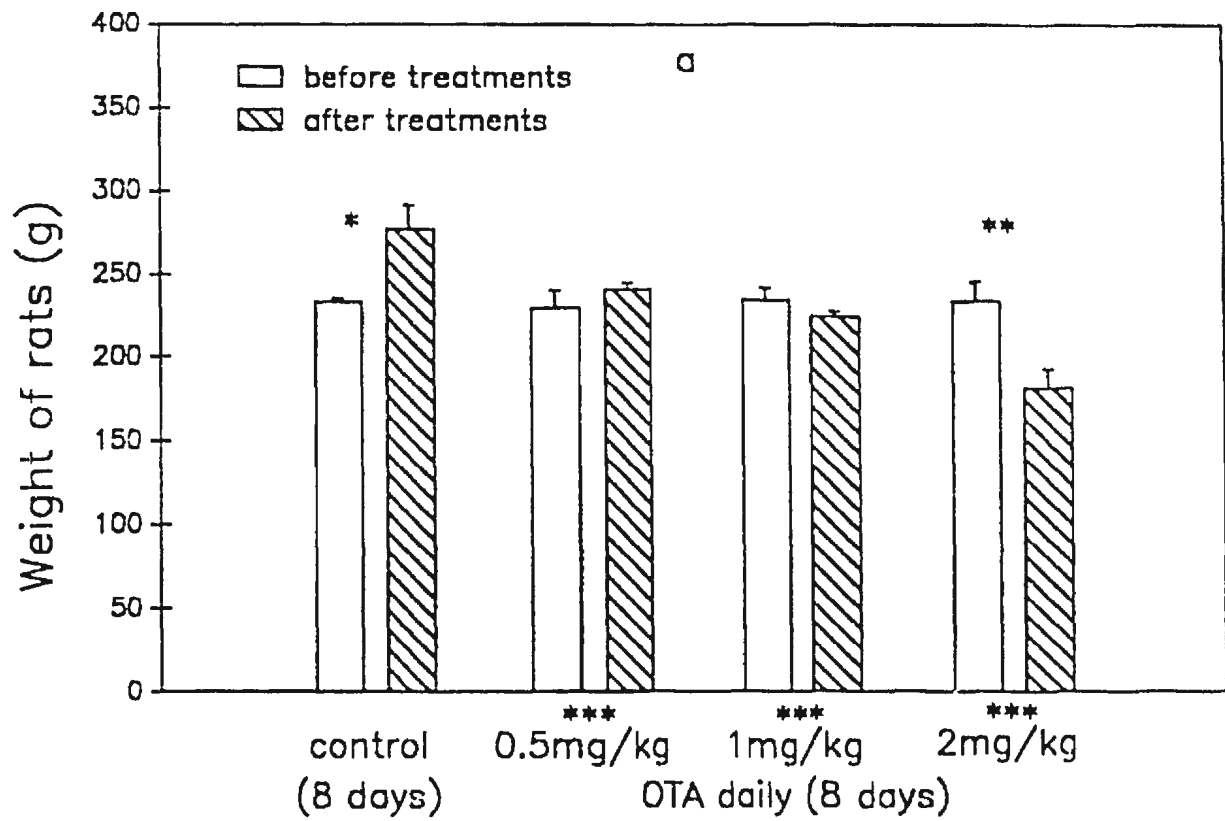


Table 2

Kidney & Liver weight as percentage of body weight

Treatment	Kidneys % bw	Liver % bw
4 days		
1. Control	0.639±0.039	3.910±0.179
2. OTA (4mg/Kg)	0.753±0.045*	3.428±0.423
8 days		
1. Control	0.820±0.040	4.757±0.167
2. OTA (0.5mg/Kg)	0.792±0.007	4.233±0.319
3. OTA (1mg/Kg)	0.848±0.006	4.687±0.165
4. OTA (2mg/Kg)	1.100±0.035***	5.863±0.142**

* denotes a significant difference ($p=0.0091$, t test)** denotes a significant difference ($p_B<0.01$, ANOVA test)*** denotes a significant difference ($p_B<0.001$, ANOVA test)

3.2 Role of lipid peroxidation

3.2.1 Lipid peroxidation in renal cortical homogenates and microsomes isolated from control and OTA treated rats

To monitor the lipid peroxidation, MDA levels were measured in renal homogenates and microsomes (Tables 3 & 4) prepared from control rats and rats dosed with OTA. As compared to controls, there was no significant change in MDA levels in either cortical homogenates or microsomes isolated from rats pretreated with 0.5 mg/Kg to 4 mg/Kg OTA, i.p., daily for 4 days, or 0.5 mg/Kg to 2 mg/Kg OTA, i.p., daily for 8 days. However, the MDA levels in cortical homogenates prepared from rats treated 30 min and 2 hr earlier with OTA (5 mg/Kg, i.p.) were 0.30 and 0.44 nmol/mg protein, which are significantly lower than the MDA level of 0.57 nmol/mg protein in cortical homogenates from control rats. A significant decrease from 0.58 nmol/mg protein to 0.46 nmol/mg protein was also observed in the cortical homogenates prepared from rats treated 2 hr earlier with OTA (10 mg/Kg, i.p.) as compared to controls. However, there was no change in the 10 min (10 mg/Kg, i.p.) treatment group.

Table 3

Effect of a single dose of OTA on renal malondialdehyde levels

Treatment	MDA (nmol/mg protein)	
	homogenate	microsomes
Control (solvent, 1h)	0.57±0.14 ¹	
5 mg/Kg (30 min)	0.30±0.02 ^{1***}	
5 mg/Kg (2 h)	0.44±0.04 ^{1**}	
Control (solvent)	0.51±0.04 ²	0.47±0.03
10 mg/Kg (10 min)	0.52±0.05 ²	0.47±0.04
control (solvent)	0.58±0.07 ²	0.46±0.01
10 mg/Kg (2 h)	0.46±0.02 ^{2*}	0.40±0.03*

Rats were treated with either solvent or OTA (i.p.). MDA levels were measured in whole homogenate or cortical homogenate or cortical microsomes as described in 2.2.5. Values shown are means ± S.D. of duplicate determinations from 3 individual rats per treatment group.

¹ whole homogenate; ² cortical homogenate

* denotes a significant difference ($p < 0.02$, t test)

** denotes a significant difference ($p_B < 0.05$, ANOVA test)

** denotes a significant difference ($p_B < 0.001$, ANOVA test)

Table 4

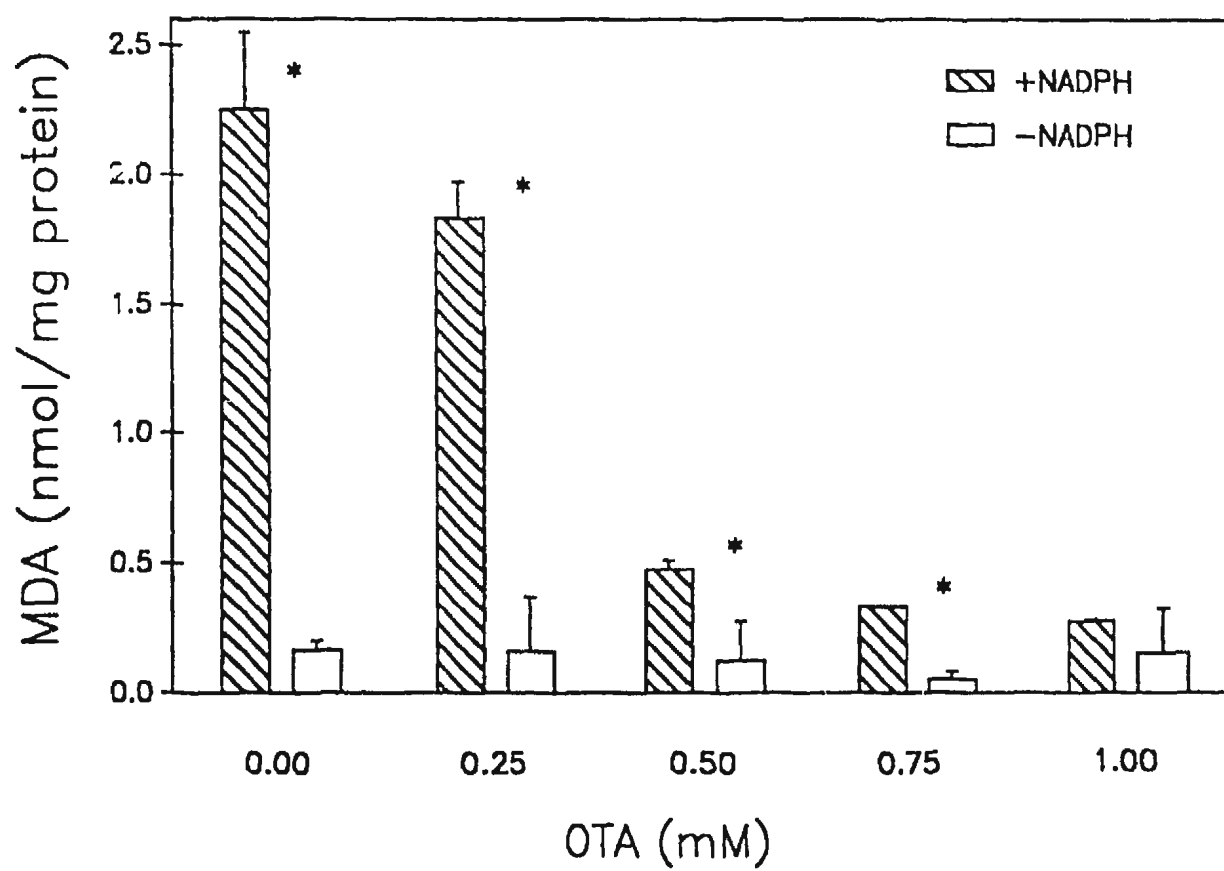
Effect of multiple doses of OTA on renal malondialdehyde levels

Treatment	MDA (nmol/mg protein)	
	homogenate	microsomes
Control (solvent)	0.25±0.06	
0.5 mg/Kg (daily for 4 days)	0.26±0.03	
1 mg/Kg (daily for 4 days)	0.25±0.01	
2 mg/Kg (daily for 4 days)	0.29±0.04	
Control (solvent)	0.23±0.02	0.39±0.07
4 mg/Kg (daily for 4 days)	0.23±0.04	0.44±0.04
Control (solvent)	0.82±0.05	0.60±0.02
0.5 mg/Kg (daily for 8 days)	0.71±0.01	0.65±0.06
1 mg/Kg (daily for 8 days)	0.64±0.08	0.57±0.04
2 mg/Kg (daily for 8 days)	0.80±0.06	0.56±0.02

Rats were treated with either solvent or OTA (i.p.). MDA levels were measured in cortical homogenate or cortical microsomes as described in 2.2.5. Values shown are means ± S.D. of duplicate determinations from 3 individual rats per treatment group.

Fig. 8. Inhibition of NADPH-dependent lipid peroxidation by OTA.

Microsomes were pre-incubated with varying concentrations of OTA \pm NADPH for 10 min. Lipid peroxidation was measured in the recovered microsomes by determining the levels of MDA as described in 2.2.5. Values shown are means \pm S.D. of two experiments (n=2), each performed in triplicate.



3.2.2 In vitro effect of OTA on renal cortical microsomal lipid peroxidation

The in vitro results (Fig. 8) are consistent with the reduced or unaltered MDA levels measured in renal cortical homogenates or microsomes after in vivo treatment. In the presence of NADPH, MDA levels were 2.25, 1.83, 0.48 and 0.33 nmol/mg protein with 0, 0.25, 0.5, 0.75 and 1.0 mM OTA respectively (Fig.8). Very little MDA was formed (< 0.2 nmol/mg protein) at the end of the incubation in the absence of NADPH. In this experiment, MDA levels were measured in parallel with calcium uptake (3.3.4 & Fig. 26) in microsomal incubations carried out in the presence of NADPH alone or NADPH along with varying concentrations of OTA. The results indicate that the observed dose-dependent reversal of NADPH inhibition of calcium uptake by OTA correlates well with the decrease in lipid peroxide levels as measured by MDA formation.

3.2.3 Studies on the protective systems against lipid peroxidation

The occurrence of oxidative stress in vivo often results in increased levels of a variety of antioxidative enzymes (Barros et al, 1991). Therefore, the levels of a variety of

cytosolic enzymes subsequent to treatment of rats with OTA were measured. The results of the 4 day treatment are shown in Table 5. The levels of GSSG reductase and superoxide dismutase (SOD) were not significantly altered from the control values of 194.4 nmol GSSG reduced and 7.8 units, respectively. Glutathione S-transferase activity was 445.3 nmol CDNB conjugated in cortical supernatants of the control rats, and dropped by about 12% (to 393.8 nmol CDNB conjugated, $p_B < 0.05$) and 21% (to 353.2 nmol CDNB conjugated, $p_B < 0.01$) in rats pretreated daily for 4 days with 0.5 mg, and 1 mg OTA/Kg respectively. The activity remained unchanged in the 2 mg/Kg and 4 mg/Kg dose groups. GSH peroxidase activity was 229.1 nmol H_2O_2 in cortical supernatants of control rats, and declined by about 26% (to 168.8 nmol H_2O_2 reduced, $p_B < 0.01$) and 31% (to 158.7 nmol H_2O_2 reduced, $p_B < 0.01$) in cortical supernatants of rats pretreated daily for 4 days with 1 mg, and 2 mg OTA/Kg respectively. The activity remained unchanged in the 0.5 and 4 mg/Kg dose groups. The levels of catalase were unaltered except in the 1.0 mg/Kg dose group, where a 29% drop (from 55.5 to 39.5 μmol , $p_B < 0.001$) was observed. The specific activity of DT-diaphorase was similarly reduced by about 26 to 38% (from 85.4 nmol to 58.9, 62.5 and 52.7 nmol in the 0.5, 1.0 and 2.0 mg/Kg doses groups respectively, $p_B < 0.05$), except in the 4.0 mg/Kg group where no reduction in the activity was observed.

Table 5

Effect of multiple OTA dosing (4 days) on activities of some antioxidant enzymes

Assay conditions are as described in 2.2.7. Values shown are means \pm S.D. of quadruplicate incubations from 3 individual rats per treatment group.

¹ $\mu\text{mol/min/mg protein}$

² $\text{nmol H}_2\text{O}_2 \text{ reduced/min/mg protein}$

³ $\text{nmol GSSG reduced/min/mg protein}$

⁴ $\text{nmol CDNB conjugated/min/mg protein}$

⁵ $\text{unit/min/mg protein, (SOD - superoxide dismutase)}$

⁶ $\text{nmol DCPIP reduced/min/mg protein}$

* denotes a significant difference ($p_8 < 0.05$, ANOVA test)

** denotes a significant difference ($p_8 < 0.01$, ANOVA test)

** denotes a significant difference ($p_8 < 0.001$, ANOVA test)

Enzyme Activity	OTA treatment (mg/Kg, i.p., daily)				
	0	0.5	1.0	2.0	4.0
Catalase ¹	55.5±0.4	56.4±1.2	39.5±0.6 ^{***}	57.3±2.4	-
GSH peroxidase ²	229.1±29.4	202.9±8.6	168.8±2.8 ^{**}	158.7±8.6 ^{**}	-
GSSG reductase ³	194.4±25.7	199.4±9.1	184.8±11.4	186.9±8.4	-
GSH S-transferase ⁴	445.3±11.0	393.8±8.2 [*]	353.2±13.2 ^{**}	440.6±39.8	-
SOD ⁵	7.8±1.2	8.9±0.1	8.4±0.4	8.5±0.1	-
DT-diaphorase ⁶	85.4±8.3	58.9±7.4 ^{**}	62.5±7.6 [*]	52.7±7.6 ^{**}	-
Catalase ¹	87.2±8.2	-	-	-	98.5±9.5
GSH peroxidase ²	146.7±9.9	-	-	-	162.4±15.6
GSSG reductase ³	139.2±7.7	-	-	-	137.4±6.8
GSH S-transferase ⁴	338.1±35.6	-	-	-	370.0±28.1
SOD ⁵	15.0±1.1	-	-	-	16.3±0.9
DT-diaphorase ⁶	41.8±8.1	-	-	-	41.5±4.1

Table 6

Effect of multiple OTA dosing (8 days) on activities of some antioxidant enzymes

Assay conditions are as described in 2.2.7. Values shown are means \pm S.D. of duplicate incubations from 3 individual rats per treatment group.

¹ $\mu\text{mol}/\text{min}/\text{mg}$ protein

² $\text{nmol H}_2\text{O}_2$ reduced/ min/mg protein

³ nmol GSSG reduced/ min/mg protein

⁴ nmol CDNB conjugated/ min/mg protein

⁵ $\text{unit}/\text{min}/\text{mg}$ protein, (SOD - superoxide dismutase)

⁶ nmol DCPIP reduced/ min/mg protein

* denotes a significant difference ($p_8 < 0.05$, ANOVA test)

** denotes a significant difference ($p_8 < 0.01$, ANOVA test)

** denotes a significant difference ($p_8 < 0.001$, ANOVA test)

Enzyme Activity	OTA treatment (mg/Kg, i.p., daily)			
	0	0.5	1.0	2.0
Catalase ¹	49.4±2.0	36.5±8.1 ^{**}	28.5±0.1 ^{***}	17.3±0.8 ^{***}
GSH peroxidase ²	219.2±42.6	194.9±31.2	241.1±22.9	221.2±28.5
GSSG reductase ³	102.5±11.3	95.5±1.4	113.8±15.6	101.5±12.8
GSH S-transferase ⁴	265.5±21.04	222.7±5.5 [*]	237.5±17.7	199.0±36.7 ^{**}
SOD ⁵	8.5±0.4	8.3±0.3	8.2±0.2	8.5±0.1
DT-diaphorase ⁶	91.1±7.4	82.2±8.2	96.4±5.1	101.1±9.5

The results of the 8 day treatment groups are shown in Table 6. There were no changes in the activities of GSSG reductase, GSH peroxidase, SOD, and DT-diaphorase. The activity of GSH S-transferase decreased significantly ($p_B < 0.05$) from the control value of 265.5 nmol CDNB conjugated to 222.7 and 199.0 in the 0.5 mg/Kg and 2.0 mg/Kg dose groups, respectively. The decrease of enzyme activity in the 1.0 mg/Kg dose group (237.5 nmol CDNB conjugated) was not statistically significant. The catalase activity dropped significantly from the control value of 49.4 μ mol to 36.5 μ mol in the 0.5 mg/Kg dose group ($p_B < 0.01$), 28.5 μ mol in the 1 mg/Kg dose group ($p_B < 0.001$), and 17.3 μ mol in the 2 mg/Kg ($p_B < 0.001$) dose group.

3.3 Rat renal cortical microsomal calcium pump studies

3.3.1 The effect of a single high dose of OTA on renal cortical microsomal calcium pump activity

Administration of a single high dose of OTA (10 mg/Kg; i.p.) resulted in a significant elevation of ATP-dependent calcium pump activity in renal cortical microsomes. Figs. 9 to 13 show the rate of calcium uptake by renal cortical

Fig. 9. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA 10 min earlier (10 mg/Kg i.p.).

Calcium uptake measurements were carried out in imidazole-histidine buffer (pH 6.8) containing 0.1 - 0.15 mg/mL microsomal protein, 5 mM MgCl₂, 5 mM ammonium oxalate, 5 mM ATP, 20 μ M CaCl₂, and 0.1 μ Ci ⁴⁵CaCl₂. At the indicated times, 0.5 mL aliquots were filtered through glass microfibre filters. The filters were washed with ice cold imidazole-histidine buffer, and bound radioactivity determined as an index of calcium uptake by microsomes. Details are described in 2.2.4.1. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 4 individual rats (n=4).

* denotes a significant difference ($p \leq 0.01$) from control.

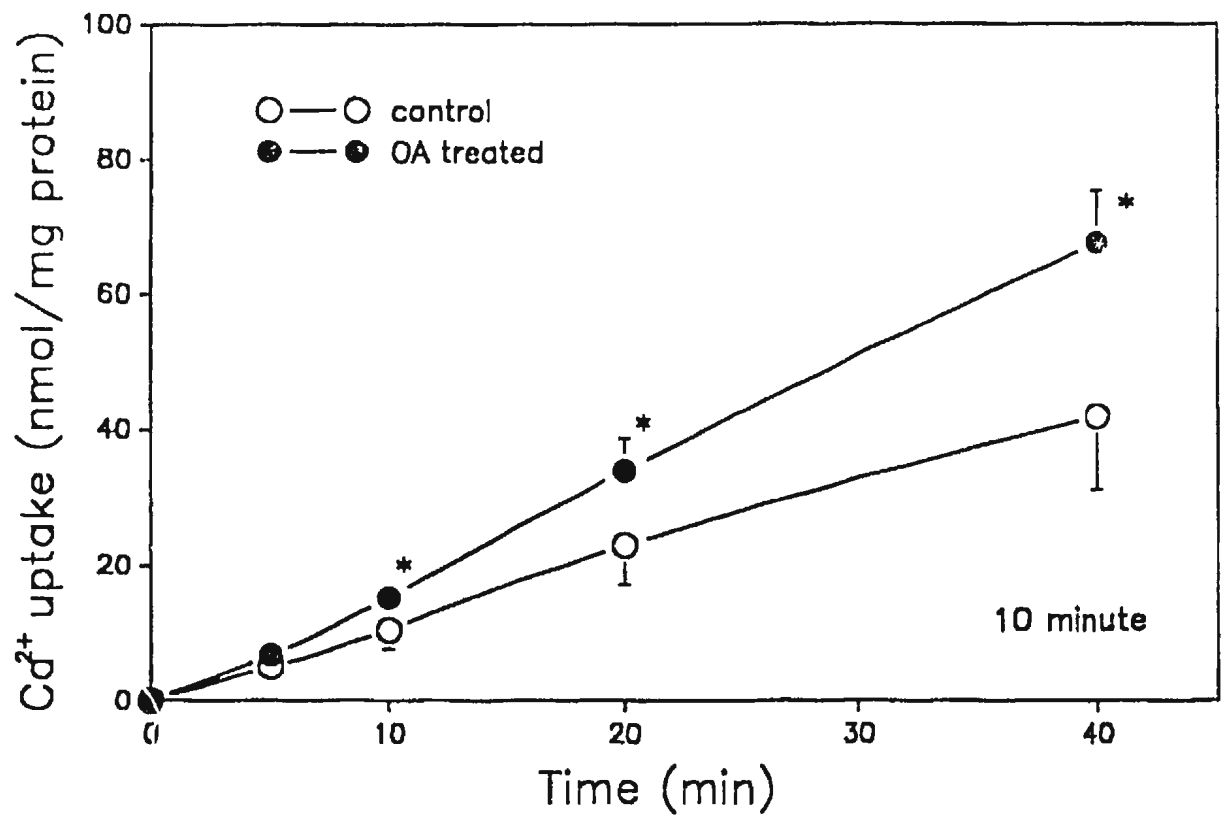


Fig. 10. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated 2 hr earlier with OTA (10 mg/Kg i.p.).

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 5 individual rats.

* denotes a significant difference ($p \leq 0.005$) from control.

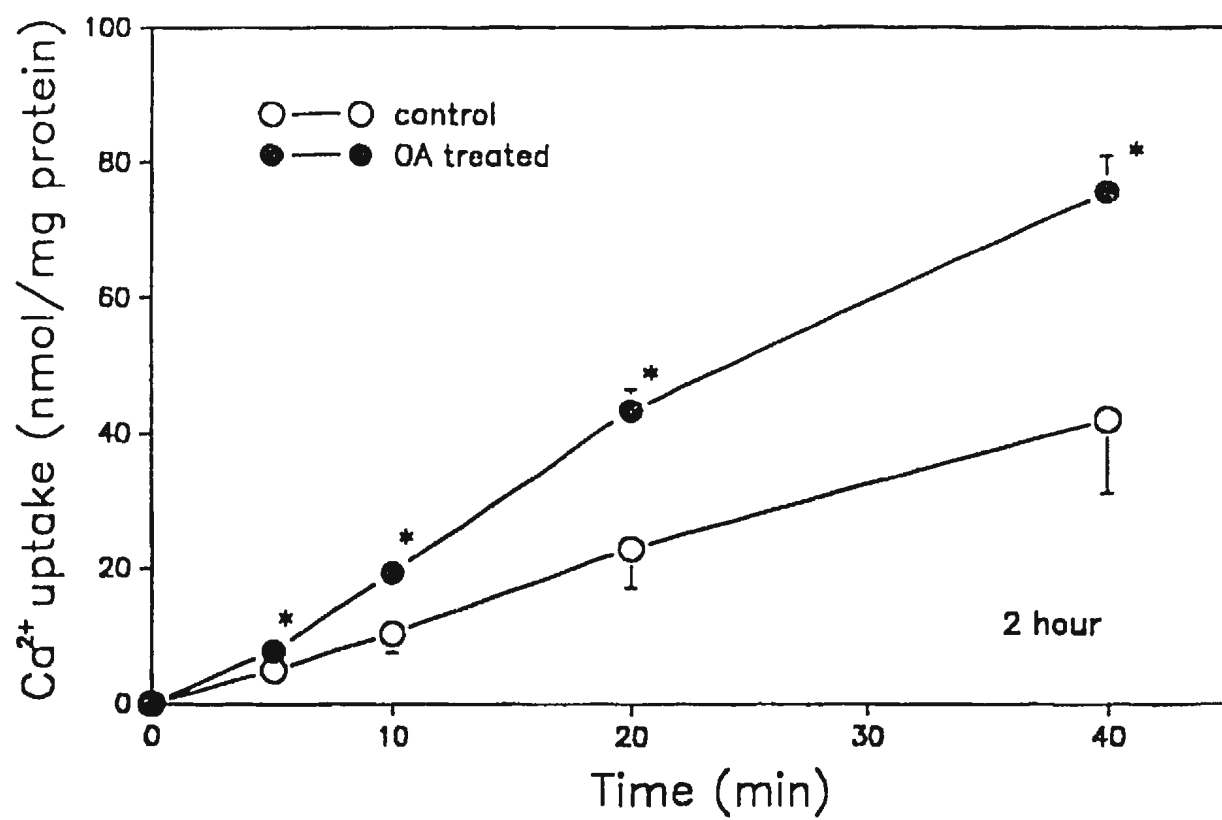


Fig. 11. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated 6 hr earlier with OTA (10 mg/Kg i.p.).

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 6 individual rats.

* denotes a significant difference ($p \leq 0.05$) from control.

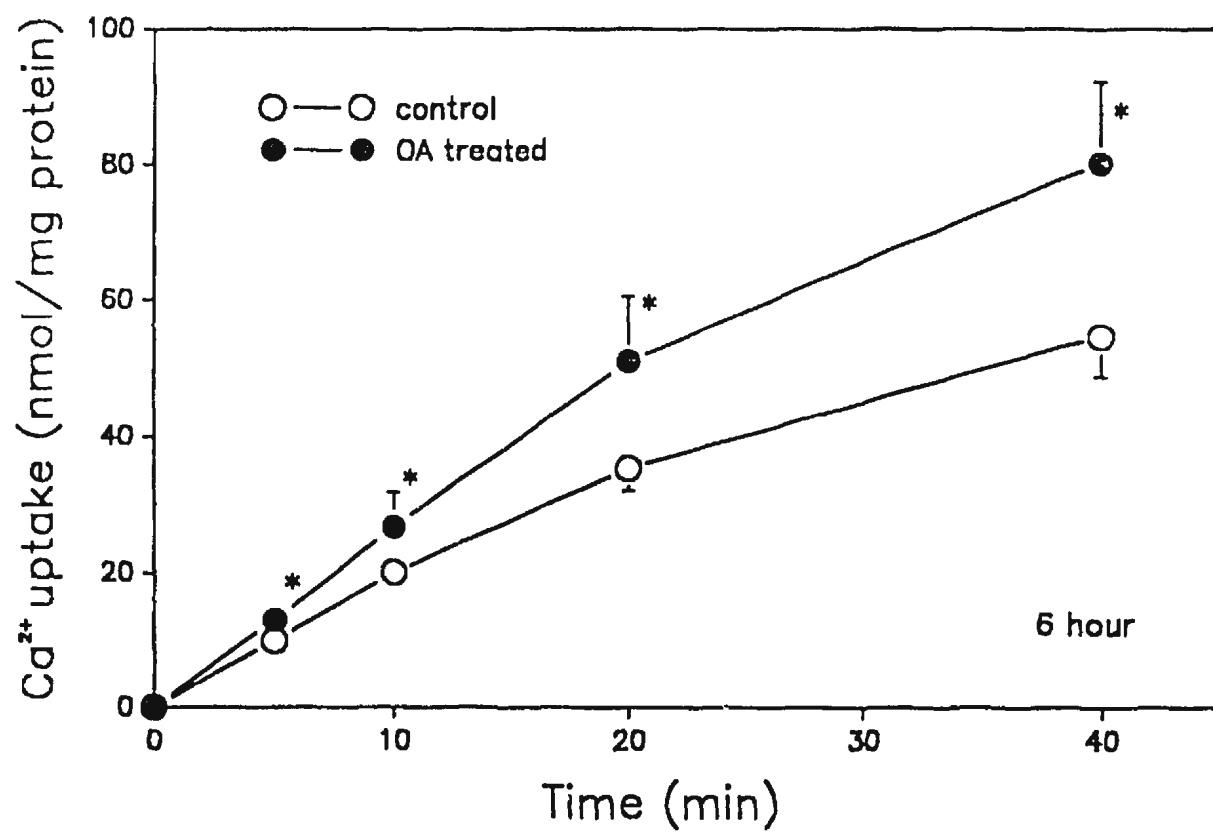


Fig. 12. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated 12 hr earlier with OTA (10 mg/Kg i.p.).

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 4 individual rats.

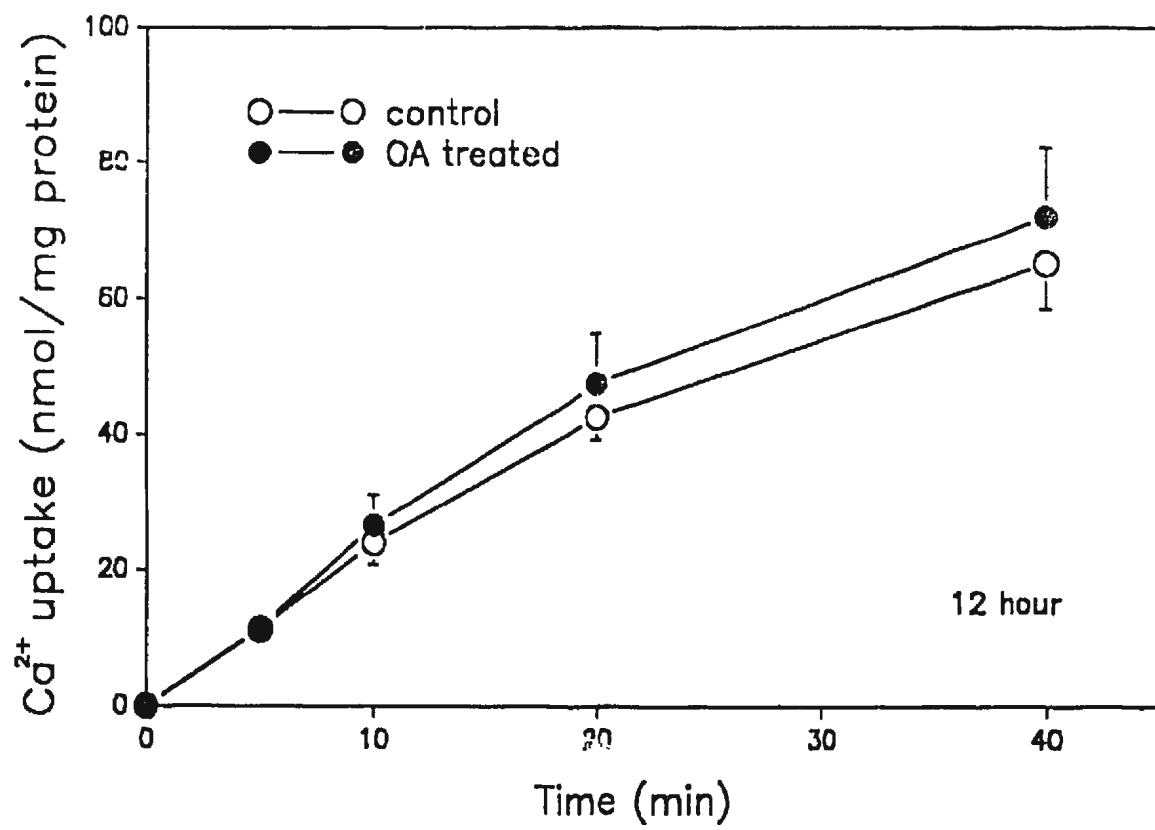
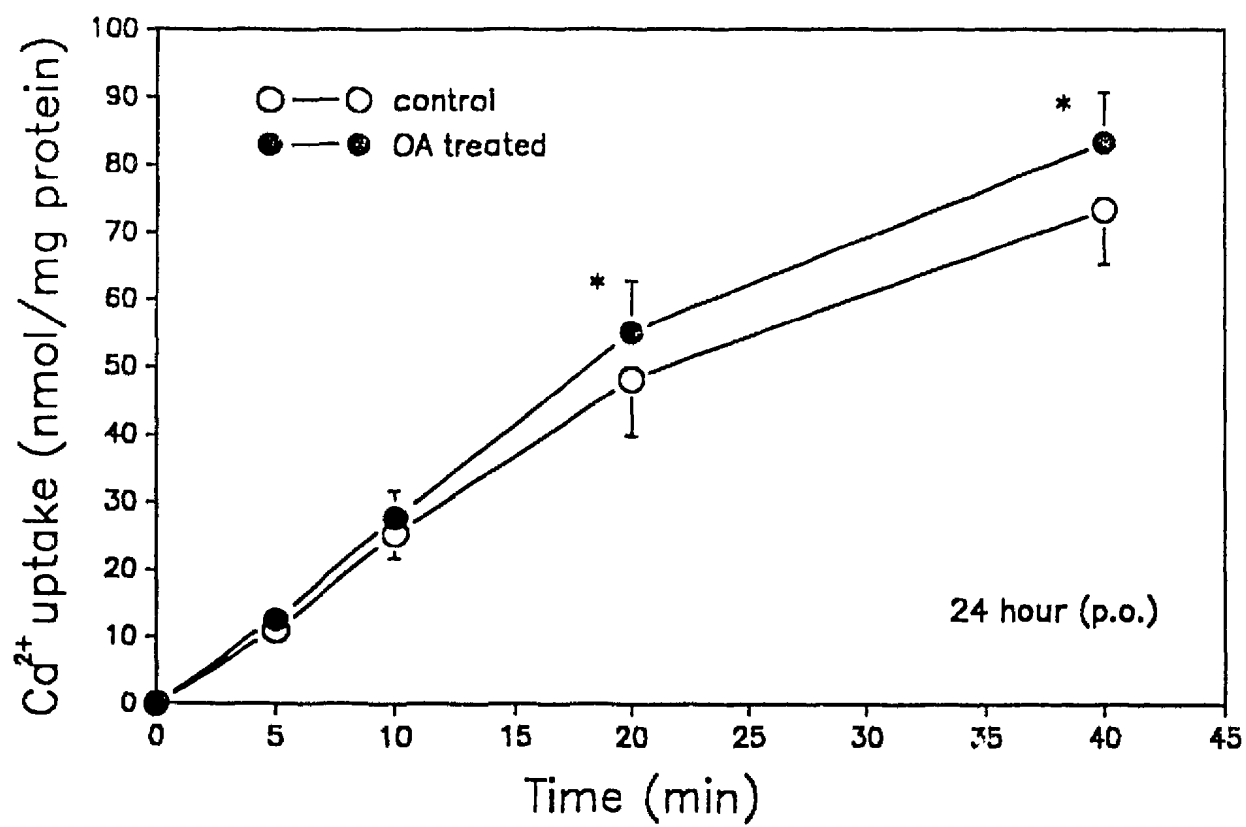


Fig. 13. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated 24 hr earlier with OTA (10 mg/Kg p.o.).

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 6 individual rats.

* denotes a significant difference ($p \leq 0.05$) from control.



microsomes isolated from rats treated 10 min, 2 hr, 6 hr, and 12 hr earlier with OTA (10 mg/Kg, i.p.) or 24 hr earlier with OTA (10 mg/Kg, p.o) or vehicle. The rate of calcium uptake was linear for about 20 min but continued to increase throughout the 40 min incubation. The elevation of calcium uptake was apparent within 10 min of OTA administration (i.p.). The rate of calcium uptake increased from about 21 nmol/20 min in the control group to about 34 nmol/20 min in the OTA dose group (Fig. 9). A maximal increase ($p < 0.005$) of about 80% (from 25 to 45 nmol/20 min) was observed after 2 hr of OTA administration (Fig. 10). The increase (from 35 nmol/20 min to 50 nmol/20 min, $p < 0.05$) could still be observed at 6 hr (Fig. 11). However, by 12 hr, there was no significant difference between the control and OTA treated groups (Fig. 12). In the p.o. treated rats, a significant increase ($p < 0.05$) in the rate of calcium uptake (from 49 to 55 nmol/20 min) was observed 24 hr after the treatment (Fig. 13).

3.3.2 The effect of multiple low doses of OTA on renal cortical microsomal calcium pump activity

A mixed response was observed after multiple dosing of rats with OTA. Lower doses of OTA elevated the rate of ATP-dependent calcium uptake while higher doses depressed it.

Administration of 1 or 2 mg/Kg, i.p. daily for 4 days increased the rate of calcium uptake to 22 nmol/20 min in the 1 mg/Kg dose group and to 17 nmol/20 min in the 2 mg/Kg dose group as compared to 12 nmol/20 min in the control group (Figs. 15 & 16). An increase in calcium uptake was also observed in the 0.5 mg/Kg dose group but this was not significant (Fig. 14). Daily dosing with 0.5 mg/Kg and 1 mg/Kg for 8 days did not alter the calcium pump activity (Figs. 18 & 19). In contrast, daily dosing with 2 mg/Kg (i.p.) for 8 days and 4 mg/Kg (i.p.) for 4 days resulted in a decrease of the calcium uptake activity of 48% (from 44 nmol/20 min to 23 nmol/20 min) to 50% (from 38 nmol/20 min to 19 nmol/20 min), respectively (Figs. 17 & 20).

3.3.3 The in vitro direct effect of OTA on renal cortical microsomal calcium pump activity

In in vitro studies, the presence of OTA in the incubation mixture during calcium uptake strongly inhibited the uptake reaction.

Fig. 14. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (0.5 mg/Kg, i.p.) daily for 4 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 4 treated or 6 control rats.

* denotes a significant difference ($p \leq 0.05$) from control.

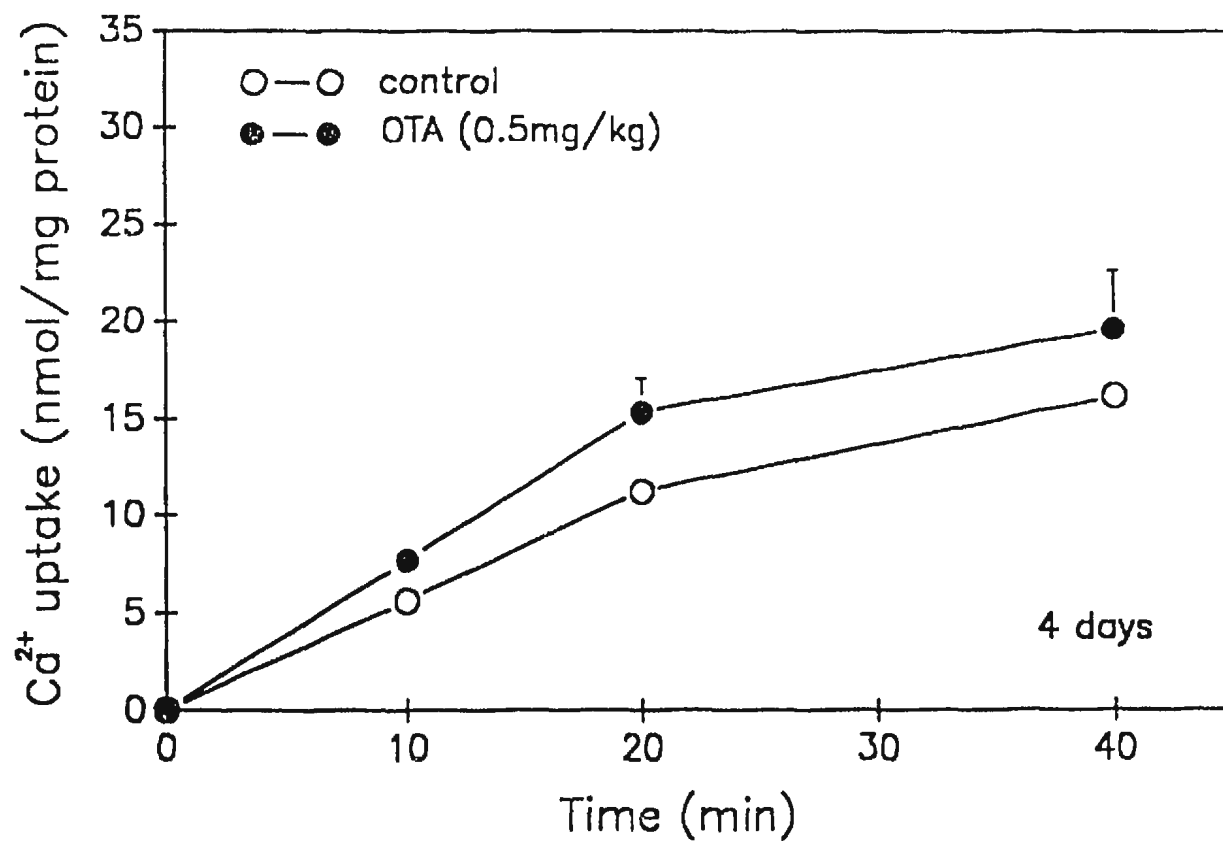


Fig. 15. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (1.0 mg/Kg, i.p.) daily for 4 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 6 control or 4 treated rats.

* denotes a significant difference ($p \leq 0.01$) from control.

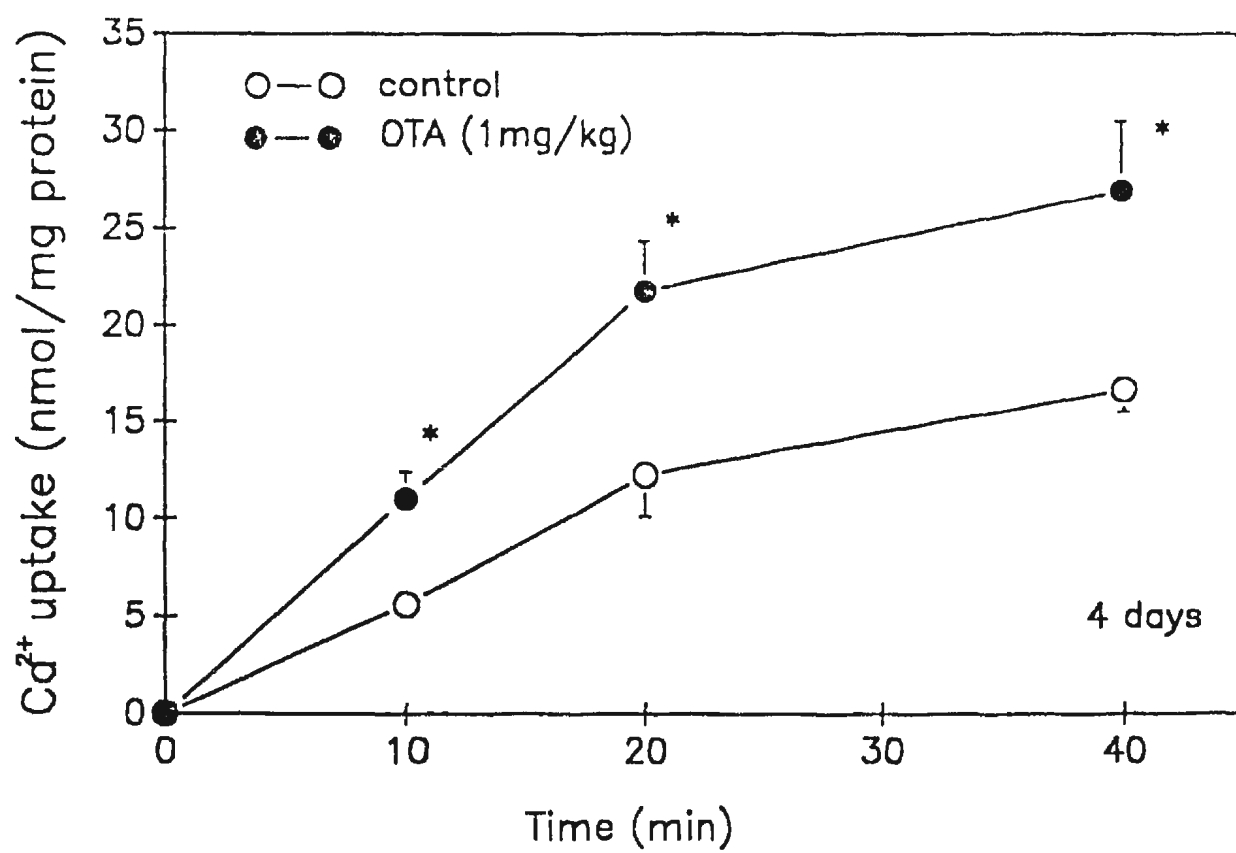


Fig. 16. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (2.0 mg/Kg, i.p.) daily for 4 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 6 control or 4 treated rats.

* denotes a significant difference ($p \leq 0.02$) from control.

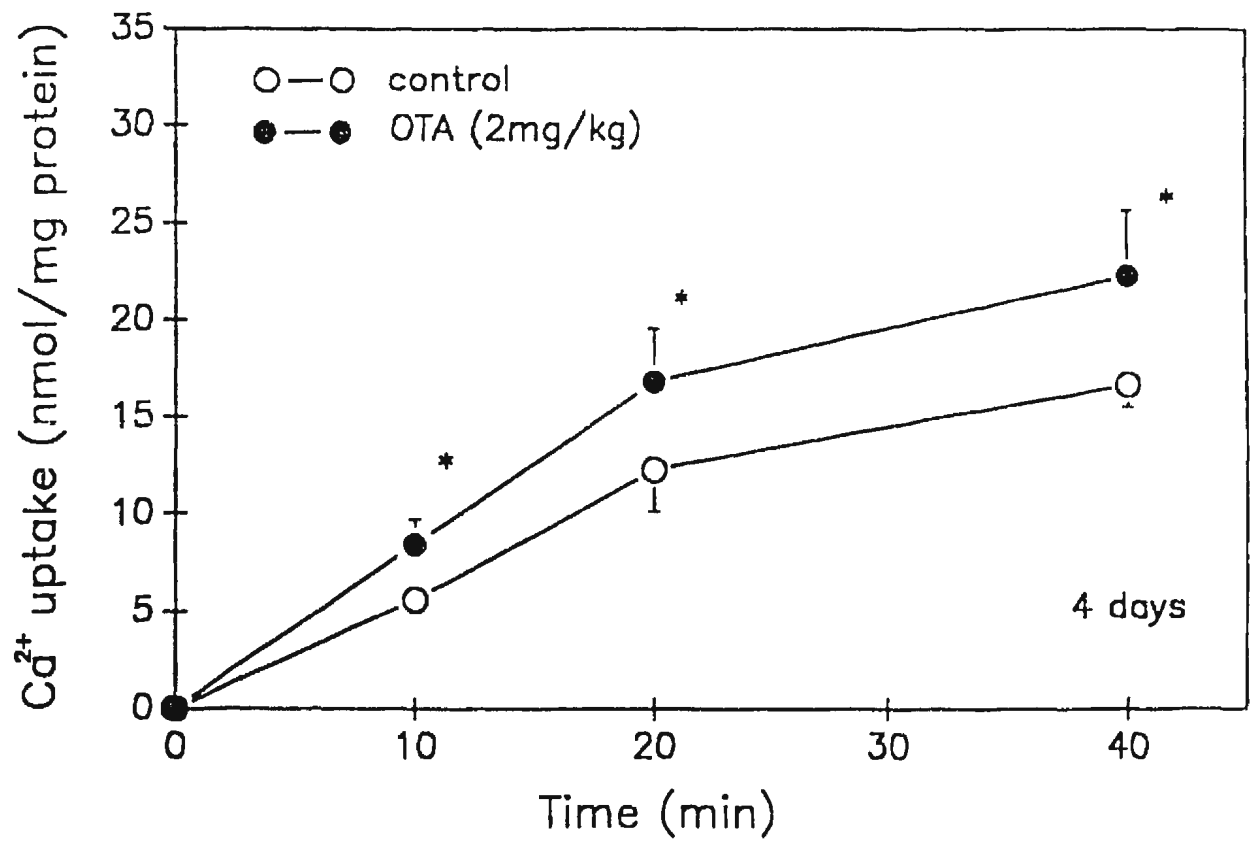


Fig. 17. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (4.0 mg/Kg, i.p.) daily for 4 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 6 individual rats.

* denotes a significant difference ($p \leq 0.0001$) from control.

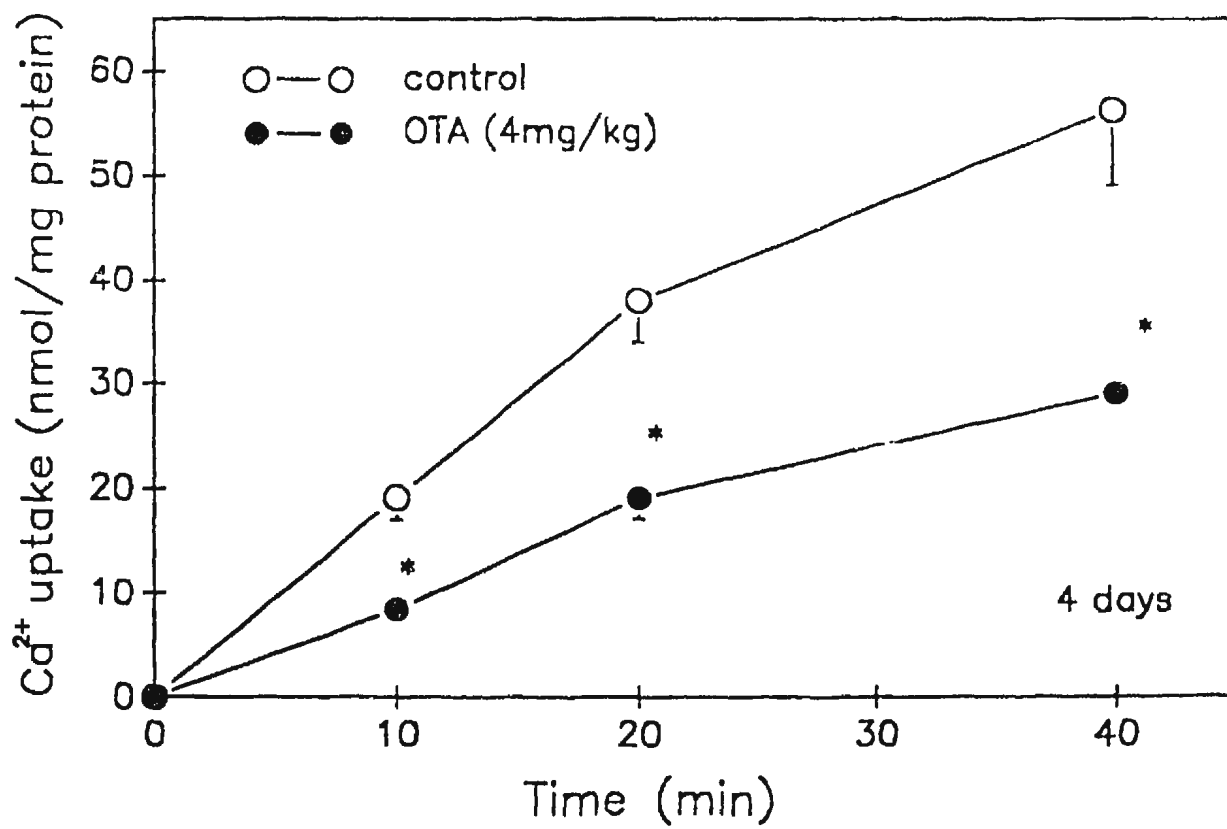


Fig. 18. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (0.5 mg/Kg, i.p.) daily for 8 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 5 individual rats.

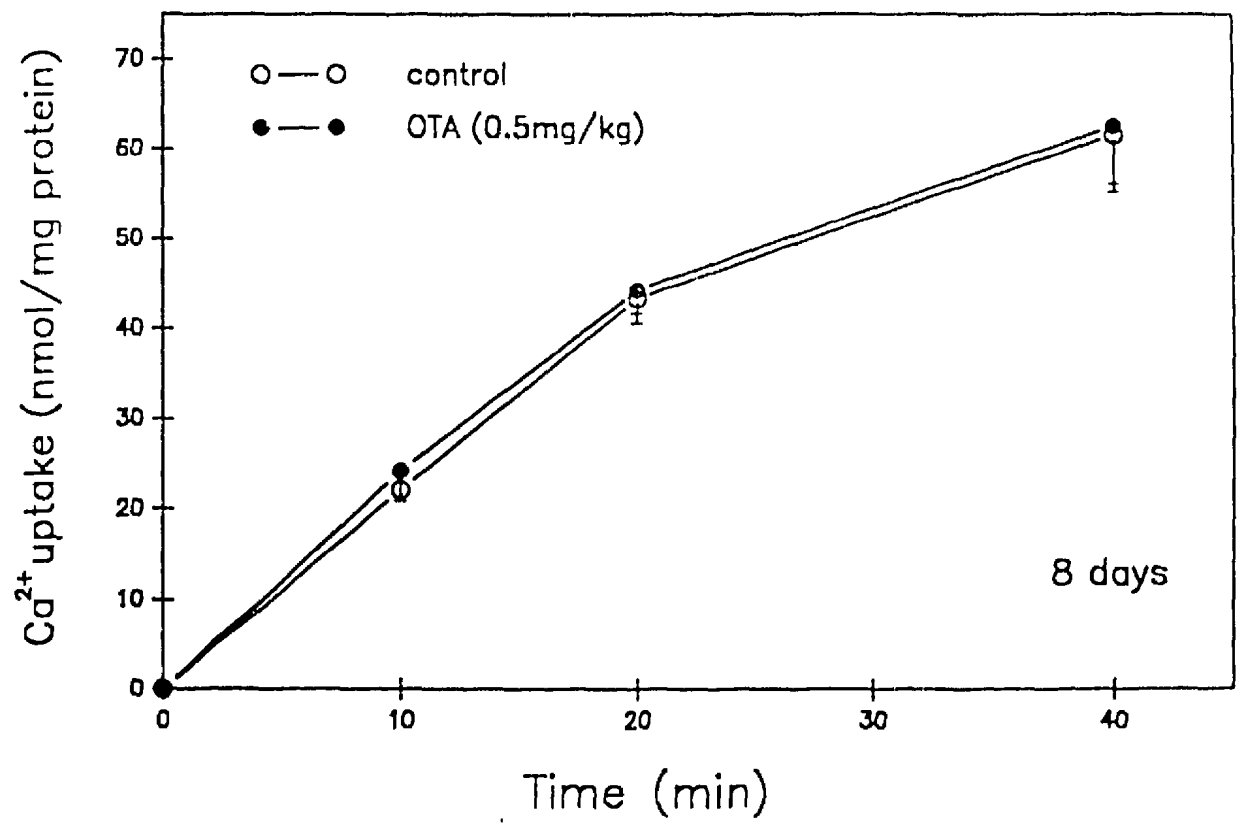


Fig. 19. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (1.0 mg/Kg, i.p.) daily for 8 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 5 individual rats.

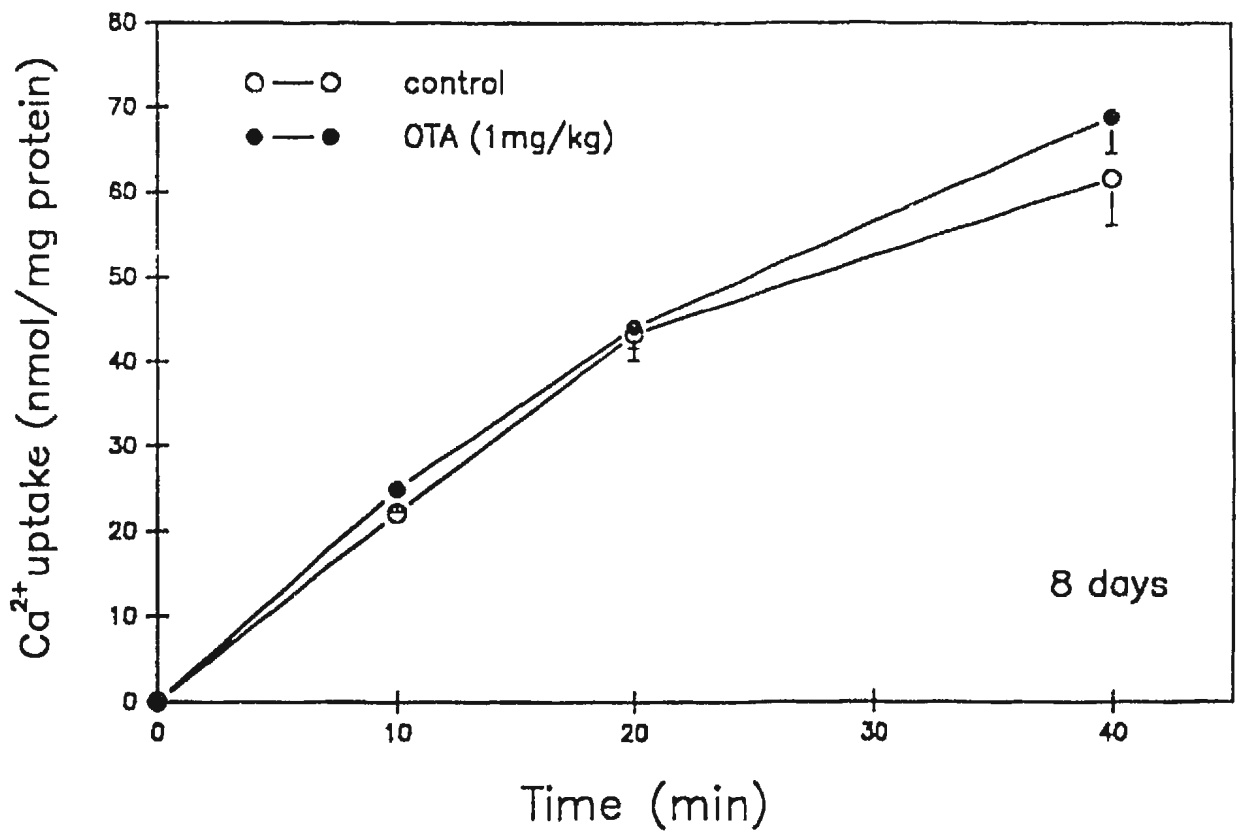
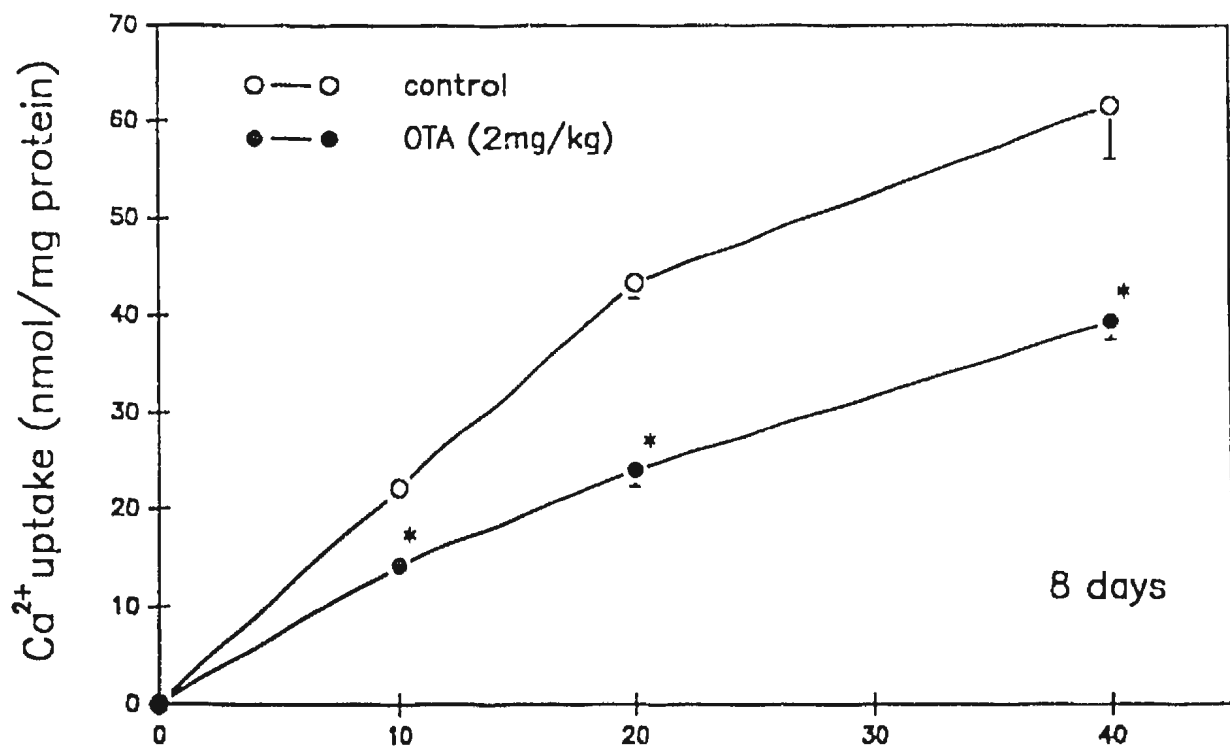


Fig. 20. Calcium uptake by renal cortical microsomes isolated from control rats and rats treated with OTA (2.0 mg/Kg, i.p.) daily for 8 days.

Calcium uptake was measured as described in Fig. 9. Microsomes from individual rats were assayed twice, each time in triplicate. Values shown are means \pm S.D. from 5 individual rats.

* denotes a significant difference ($p \leq 0.0001$) from control.



The calcium uptake rate of 65 nmol at 20 min of incubation in the absence of OTA dropped by 65% (to 23 nmol), 77% (to 15 nmol) or 98% (to 1.5 nmol) in the presence of 0.25 mM, 0.5 mM or 1 mM OTA, respectively (Fig. 21). This inhibitory effect of OTA was both rapid and (at least partially) reversible. The effect was rapid because addition of OTA (0.5 mM) 5 or 10 min after calcium uptake was initiated, resulted in an inhibition of calcium uptake similar to that observed if OTA had been present at the start of the incubation (Fig. 22). The effect appeared to be reversible because, in comparison to direct addition (Fig. 21), pre-incubation of microsomes with 0.25 mM, 0.5 mM or 1 mM OTA for 10 min followed by centrifugation, led to only a 18%, 35% or 54% inhibition of calcium uptake respectively from the control value of 44.5 nmol/40 min in recovered microsomes incubated in the absence of OTA (Fig. 23).

3.3.4 The in vitro effect of OTA on renal cortical microsomal calcium uptake in the presence of NADPH

3.3.4.1 NADPH-dependent calcium uptake

In an effort to determine if mixed function oxidase (MFO)-dependent metabolism of OTA alters its effect on

Fig. 21. Inhibitory effect of in vitro OTA addition on calcium uptake by renal cortical microsomes.

Calcium uptake was measured as described in Fig. 9 but in the presence of various concentrations of OTA. Values shown are means \pm S.D. of two experiments, each performed in triplicate.

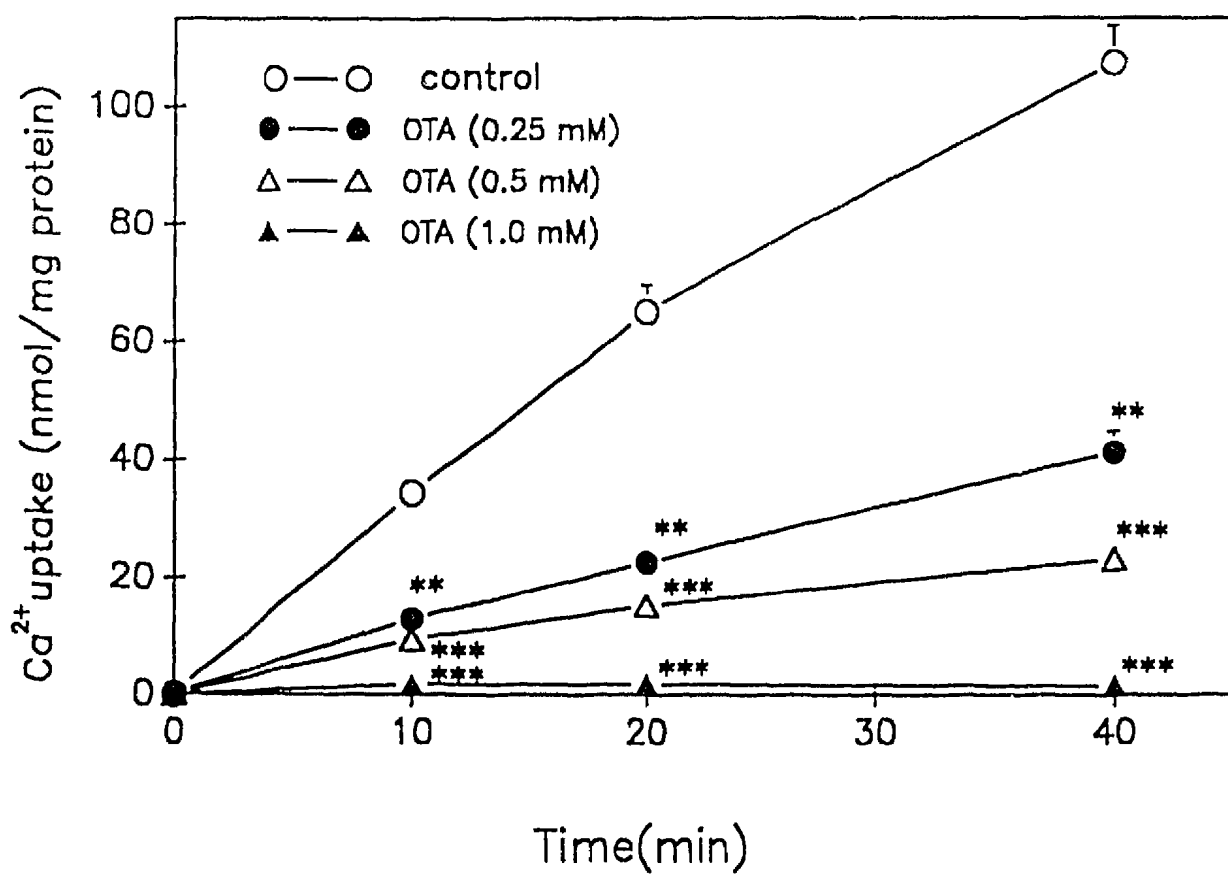


Fig. 22. Effect of delayed addition of OTA on calcium uptake by cortical microsomes.

Calcium uptake was carried out as in Fig. 9 except that OTA was added at 0 min, 5 min or 10 min after the start of the reaction. Values shown are means \pm S.D. of two experiments, each performed in triplicate.

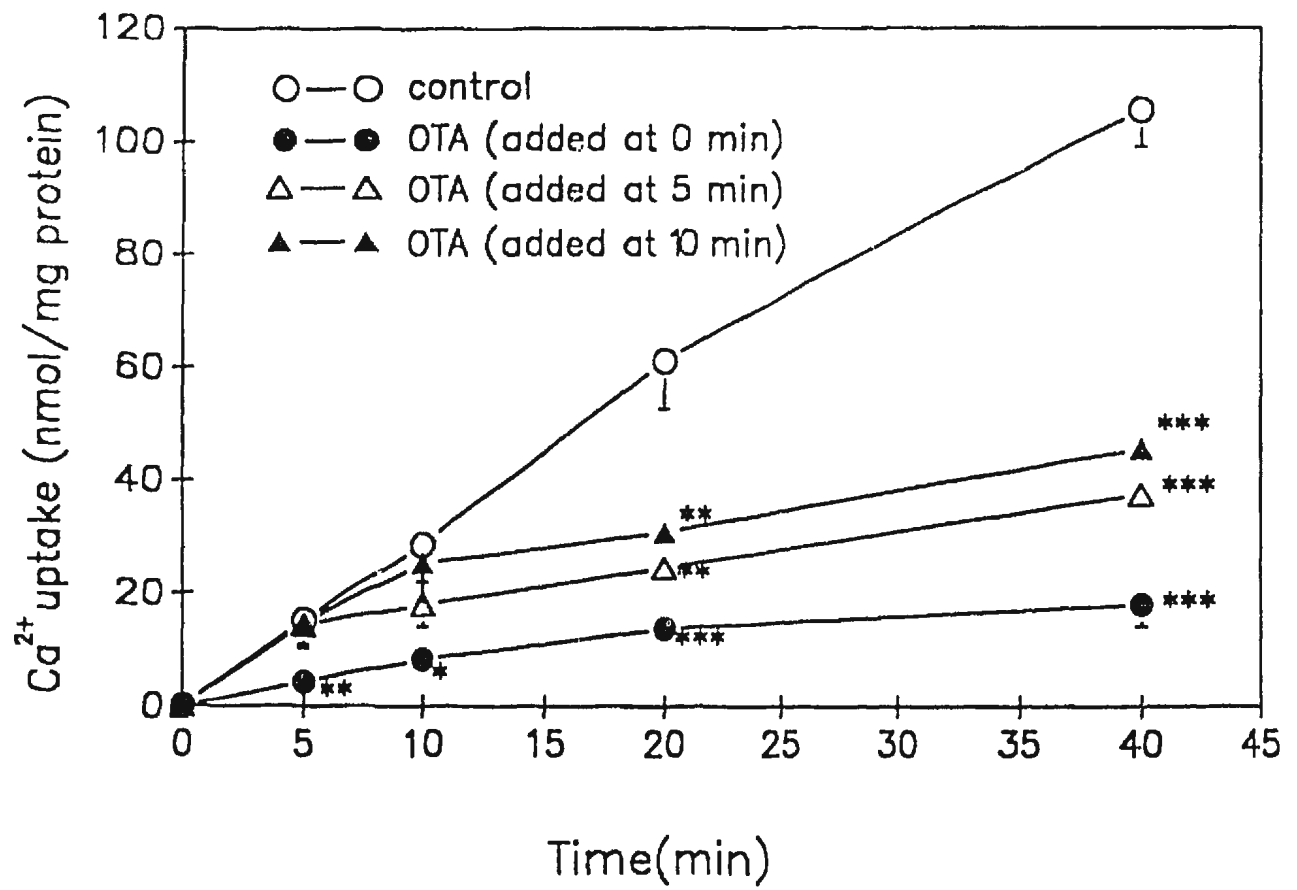
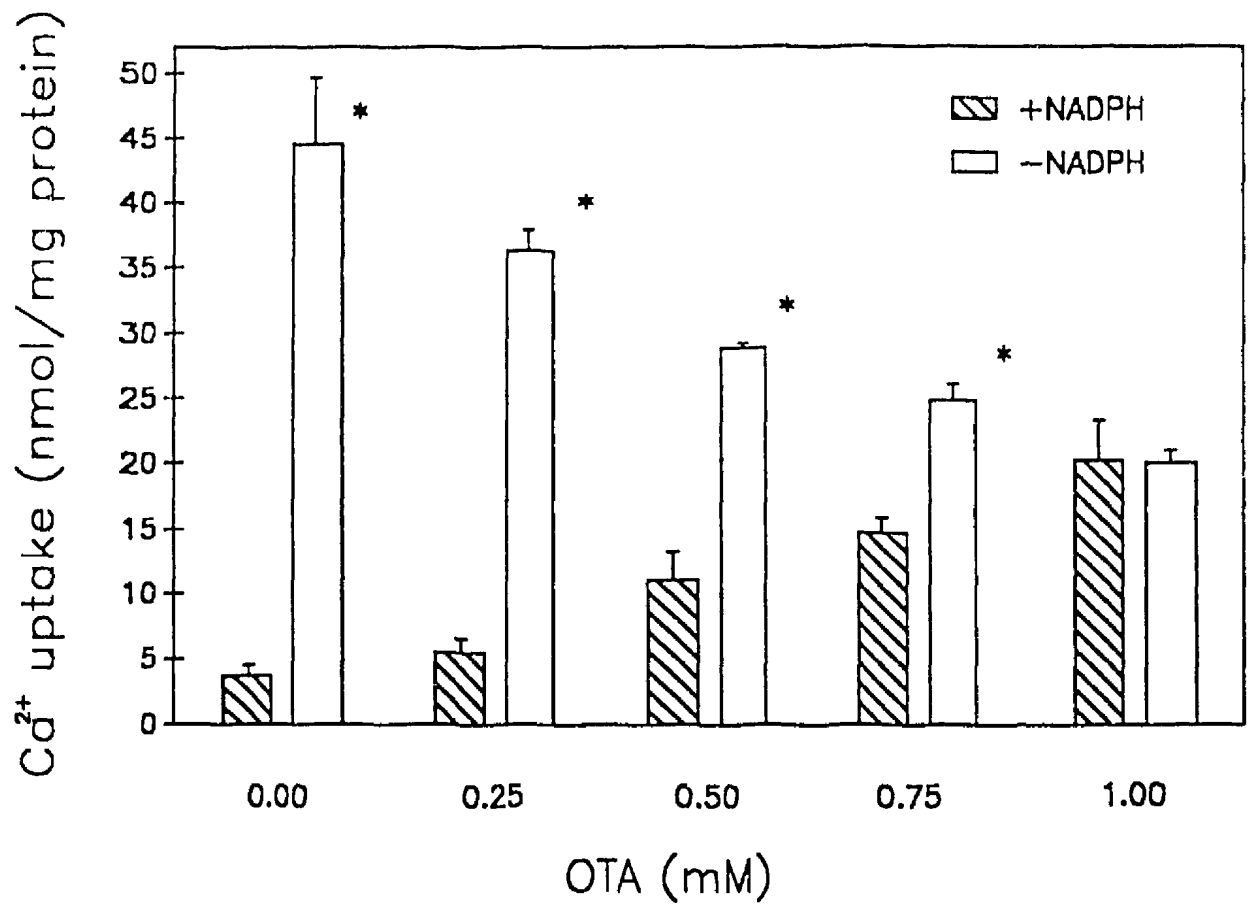


Fig. 23. Inhibition of calcium uptake by pre-incubation of microsomes with NADPH and its reversal by OTA.

Cortical microsomes were pre-incubated for 10 min with the indicated concentrations of OTA in the presence or absence of NADPH. Subsequent to centrifugation, calcium uptake by the recovered microsomes was determined as described in Fig. 9 except that only the 40 min point was taken. Values shown are means \pm S.D. of two experiments, each performed in triplicate.



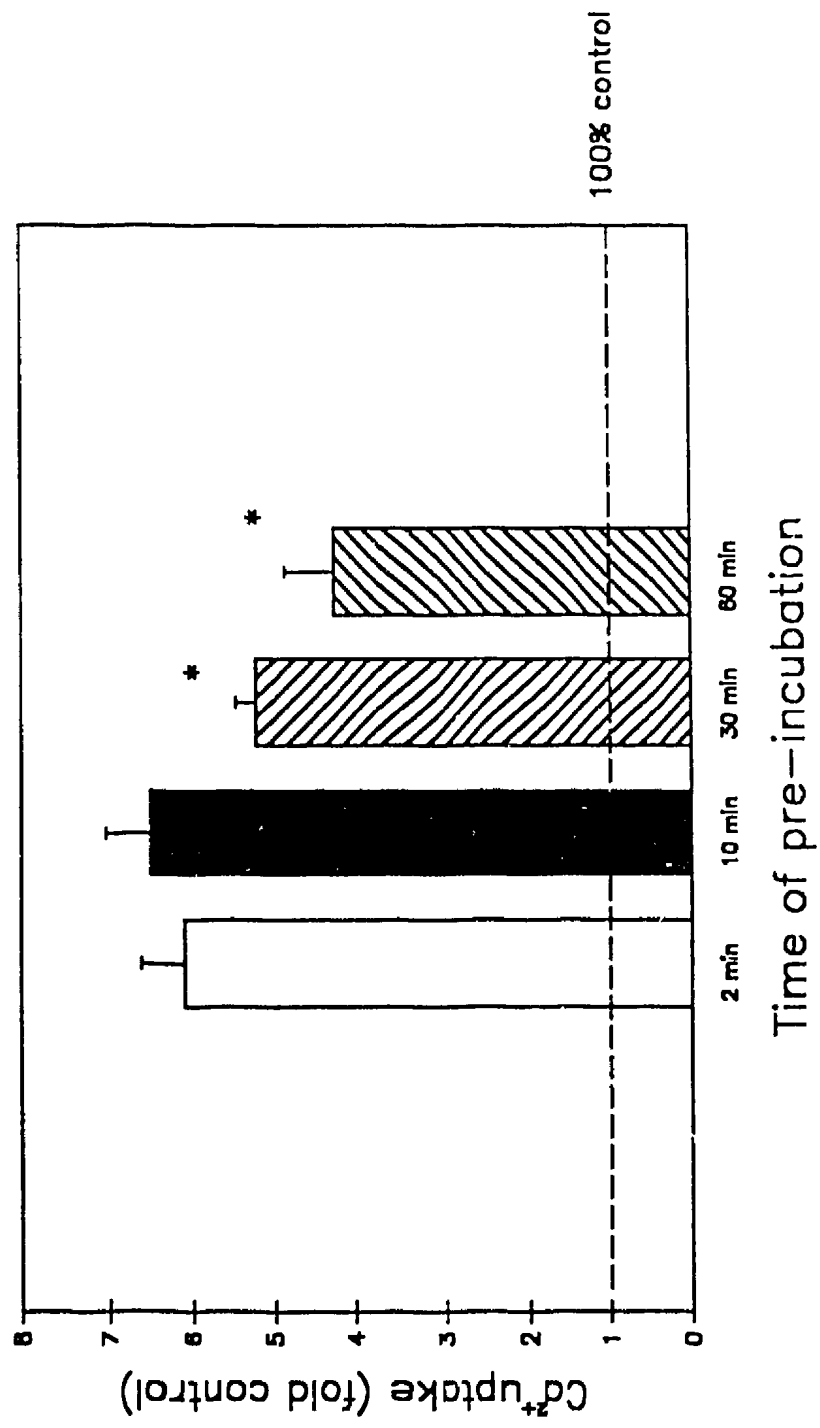
calcium uptake, NADPH was also included in the incubation of medium. Calcium uptake was measured in parallel with MDA (3.2.2, Fig. 8) in microsomes pre-incubated with varying concentrations of OTA alone or in the presence of NADPH. Results shown in Fig. 23 indicate that pre-incubation of microsomes with NADPH strongly suppressed their ability to sequester calcium (by about 92%, from 44.5 nmol/mg protein to 3.7 nmol/mg protein). However, inclusion of OTA during pre-incubation with NADPH resulted in a partial concentration-dependent reversal of this inhibitory effect. Thus, the microsomes pre-incubated with 1 mM OTA and NADPH appeared to have a 5 to 10 fold "higher" rate of calcium uptake than that observed in microsomes pre-incubated with NADPH alone.

Fig. 24 shows the effect of pre-incubation time with OTA (1 mM) in the presence of NADPH on the alterations of microsomal calcium pump activity. The "stimulatory" effect of OTA was most significant in 10 min pre-incubated microsomes (about 6.3-fold), while the "stimulatory" effect was 5.8-fold, 5.2-fold and 3.6-fold in 2 min, 30 min and 60 min pre-incubated microsomes, respectively.

Fig. 24. Effect of varying preincubation times of cortical microsomes with OTA (1 mM) and NADPH on subsequent calcium uptake activity.

Cortical microsomes were pre-incubated for 2 to 60 min with OTA in the presence of NADPH. Subsequent to centrifugation, calcium uptake by the recovered microsomes was determined as described in Fig. 9 except that only the 40 min point was taken. Values shown are means \pm S.D. of two experiments, each performed in triplicate.

* denotes a significant difference ($p < 0.05$, ANOVA).



3.3.4.2 ATP-dependent calcium uptake by rat renal cortical microsomes

Calcium uptake by rat renal cortical microsomes pre-incubated with NADPH in the presence or absence of OTA was always measured with ATP present (5 mM). In order to demonstrate the dependence of calcium uptake on ATP, the later was omitted from two of the incubations in this experiment. Fig. 25 shows that in the presence of ATP, OTA strongly "stimulated" calcium uptake by about 5-fold (from 1.5 to 8 nmol/20 min), but in the absence of ATP there was almost no calcium uptake by either control or OTA pre-incubated microsomes.

3.3.4.3 Oxalate-dependent calcium uptake by rat renal cortical microsomes

Oxalate is normally used to enhance calcium uptake in hepatic microsomes, as a result of its permeation into the lumen and the formation of an insoluble calcium complex [Parys et al, 1985; Heilmann et al, 1984]. This experiment was set up to demonstrate the enhancement of calcium uptake by oxalate in renal microsomes. The results show (Fig. 26) that in the control samples in the presence of NADPH, the renal cortical microsome calcium uptake in the absence of oxalate was much

Fig. 25. Role of ATP on renal cortical microsomal calcium uptake.

Calcium uptake was measured as described in Fig. 9 in the presence or absence of ATP. Values shown are means \pm S.D. of two experiments, each performed in triplicate.

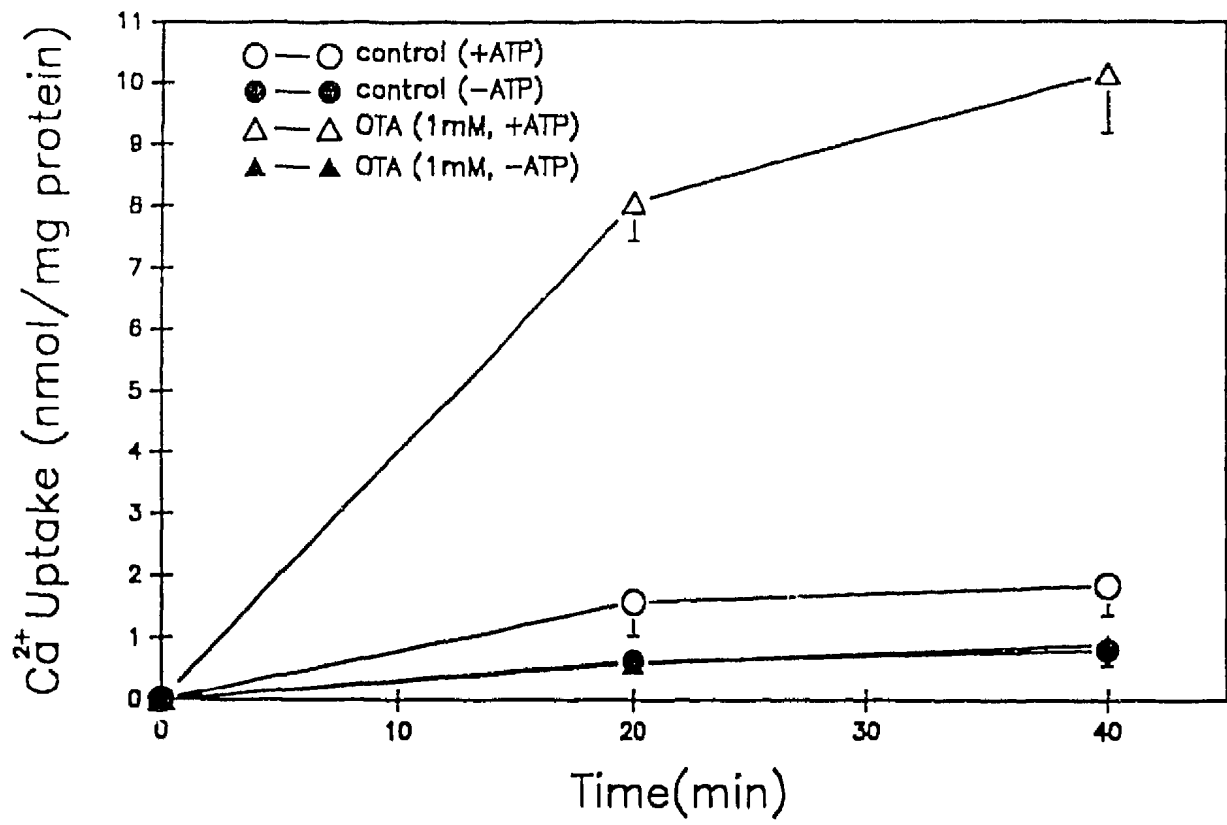
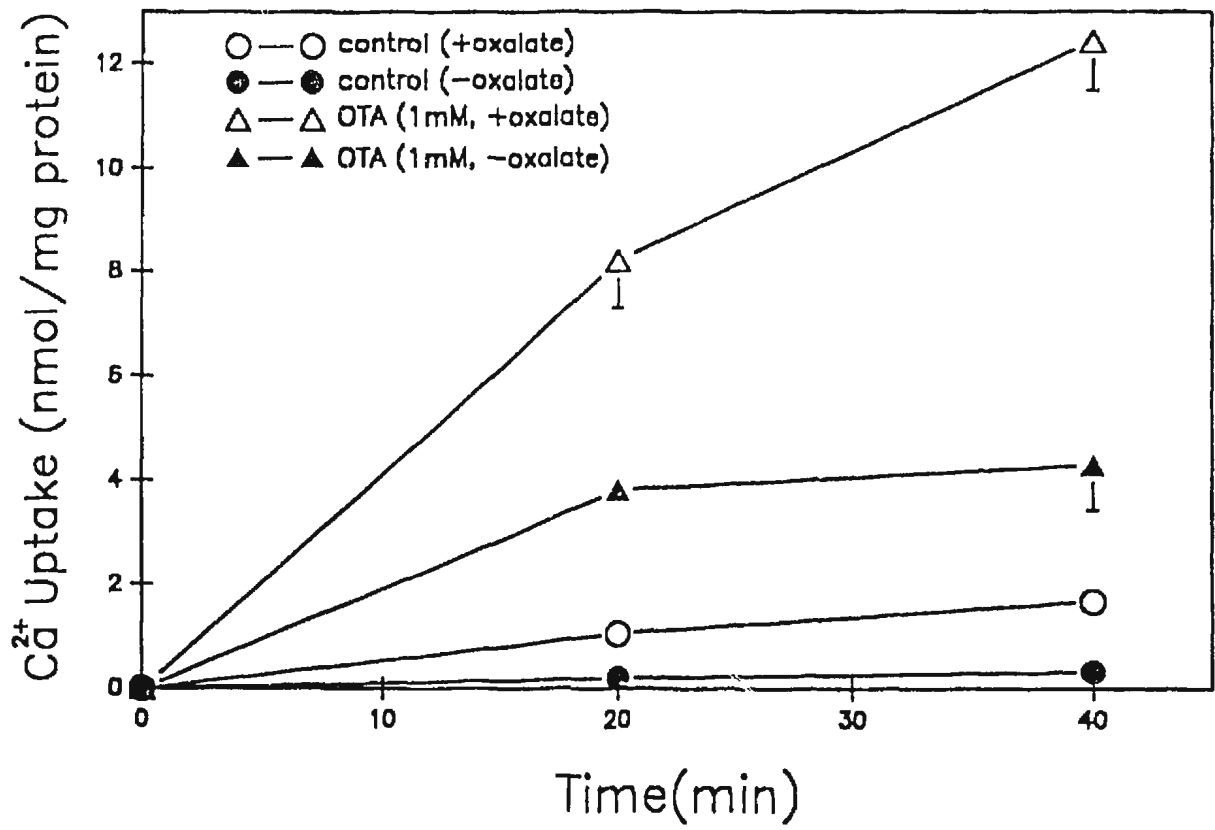


Fig. 26. Role of ammonium oxalate on renal cortical microsomal calcium uptake in the presence of NADPH \pm OTA.

Calcium uptake was measured as described in Fig. 9, but in the presence or absence of ammonium oxalate (5 mM). Values shown are means \pm S.D. of two experiments, each performed in triplicate.



lower (0.2 nmol/20 min) than that in the presence of oxalate (1.5 nmol/20 min). Also, in the samples pre-incubated with OTA in the presence of NADPH, calcium uptake in the absence of oxalate was much lower (3.8 nmol/20 min) than that in the presence of oxalate (8.4 nmol/20 min). In both cases, with and without oxalate, the calcium uptake was significantly "enhanced" by OTA.

3.3.4.4 Dependence of calcium uptake on calcium ion concentration

Fig. 27a shows that calcium uptake "stimulated" by OTA in the presence of NADPH was dependent on the calcium ion concentration. Fig. 27b shows the double reciprocal plot (Lineweaver-Burk plot) [Armstrong, 1983] of $1/V$ vs. $1/[Ca^{2+}]$. The two intercepts on the vertical axis were different, which indicates that the V_{max} of the reaction was altered by OTA. However, the two intercepts on the horizontal axis were the same indicating that the K_m of the ATPase for calcium was not changed by OTA.

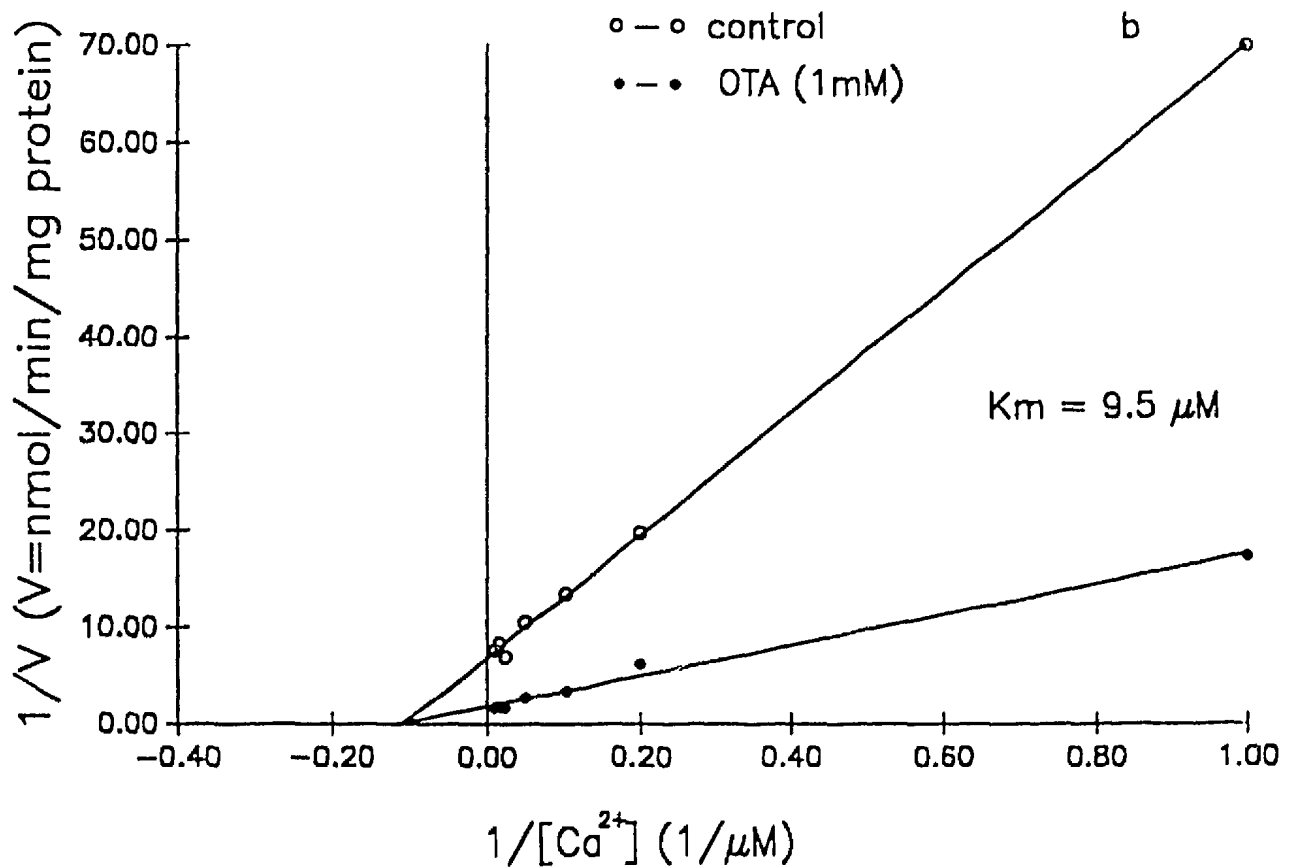
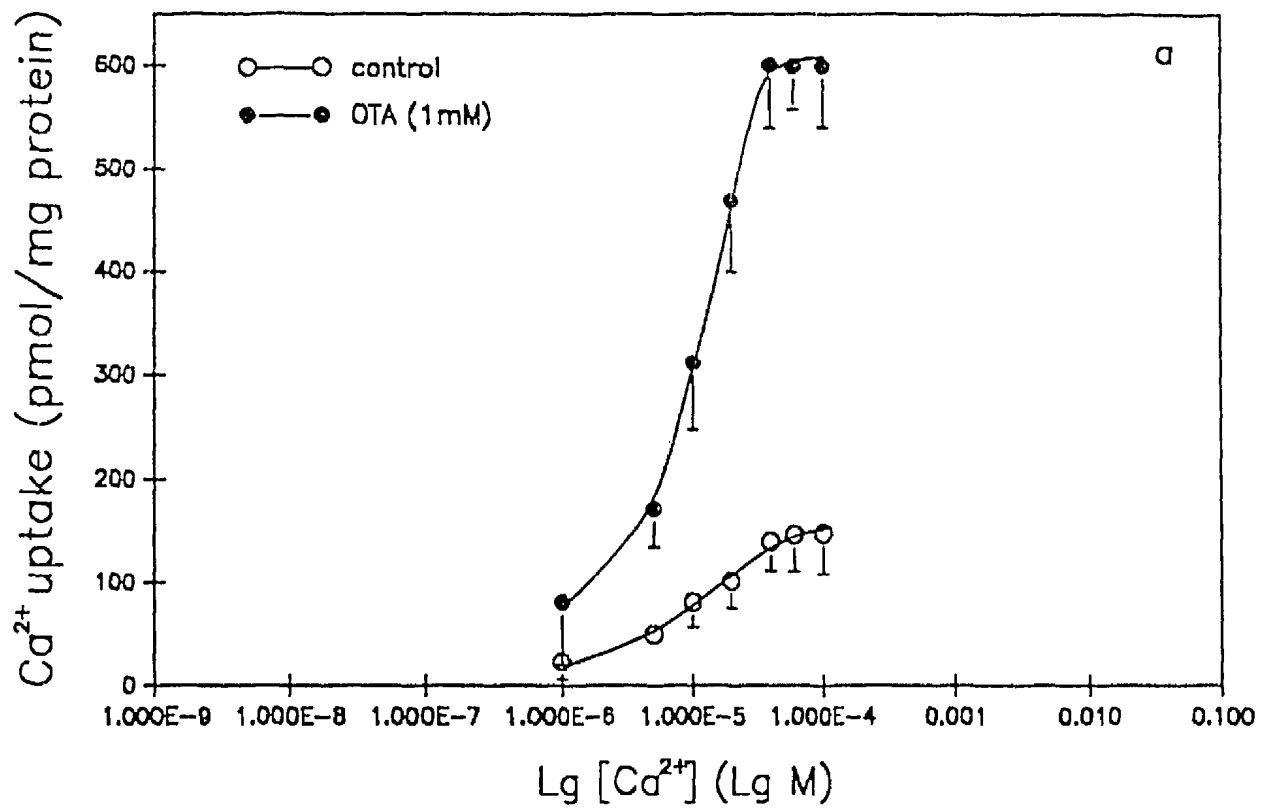
3.3.5 The effect of OTA on the levels of the phosphoenzyme intermediate

In the presence of calcium, addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to

Fig. 27a. Dependence of calcium uptake on the concentration of Ca^{2+} .

Fig. 27b. Double Reciprocal Plot (Lineweaver-Burk Plot) of Fig. 27a.

Calcium uptake was measured as described in Fig. 9, but with the various concentration of CaCl_2 . Values shown are means \pm S.D. of two experiments, each performed in triplicate.



hepatic microsomes is followed by rapid formation of the phosphorylated enzyme intermediate [Spamer et al, 1987]. The results show that alterations in the rate of calcium uptake brought about by in vivo/in vitro OTA treatments correlated well with the levels of the phosphoenzyme intermediate (Table 7). Thus, OTA administration which led to an increased rate of ATP-dependent microsomal calcium uptake also showed a

Table 7

Effect of OTA on phosphorylation of renal microsomes

Treatment		[³² P] bound (nmol/mg protein X 10 ⁶)	
		Expt 1	Expt 2
<u>In vivo</u>			
1.	Control	0.7	0.6
2.	OTA (10mg/Kg, i.p., 2hr)	1.7	1.5
3.	Control	7.0	5.8
4.	OTA (4mg/Kg daily for 4 days)	2.9	2.5
<u>In vitro</u>			
1.	Control (- NADPH)	1.2	1.4
2.	+ NADPH	0.3	0.2
3.	OTA (1mM)	0.6	0.7
4.	OTA (1mM) + NADPH	0.8	0.6

Microsomes were labelled with [γ -³²P]ATP and subjected to SDS-PAGE as described in 2.2.6. Individual values from two separate experiments are shown.

higher level of the phosphoenzyme intermediate (1.6×10^{-6} nmol) as compared to control microsomes (0.65×10^{-6} nmol).

Conversely, reversal by OTA of the NADPH-dependent inhibition of calcium uptake was also accompanied by a simultaneous increase in the level of the phosphoenzyme intermediate (from 0.25×10^{-6} nmol to 0.7×10^{-6} nmol).

3.3.6 The role of calcium backflux in the effect of OTA on renal cortical microsomes

The net rate of calcium uptake by the ER is the difference between the rate of calcium uptake and the calcium release (backflux) by the ER membrane. Thus, an alteration in the calcium backflux rate by OTA could contribute to the difference in calcium uptake rates observed in the presence of OTA. However, addition of OTA (up to a concentration of 1 mM) to microsomes loaded with ^{45}Ca did not significantly alter the rate of calcium backflux (Fig 28).

3.3.7 ATP consumption during calcium uptake

Since calcium uptake is dependent on the presence of ATP, it was important to demonstrate that an inhibition of calcium uptake was not due to premature depletion of ATP. Hence, ATP consumption during calcium uptake was measured in order to

determine if the added ATP was sufficient for calcium uptake. The results show that at the end of the incubation (40 min), up to 35% of ATP was consumed in the OTA/NADPH addition group, and about 10% of ATP was used in the NADPH control group (Fig. 29).

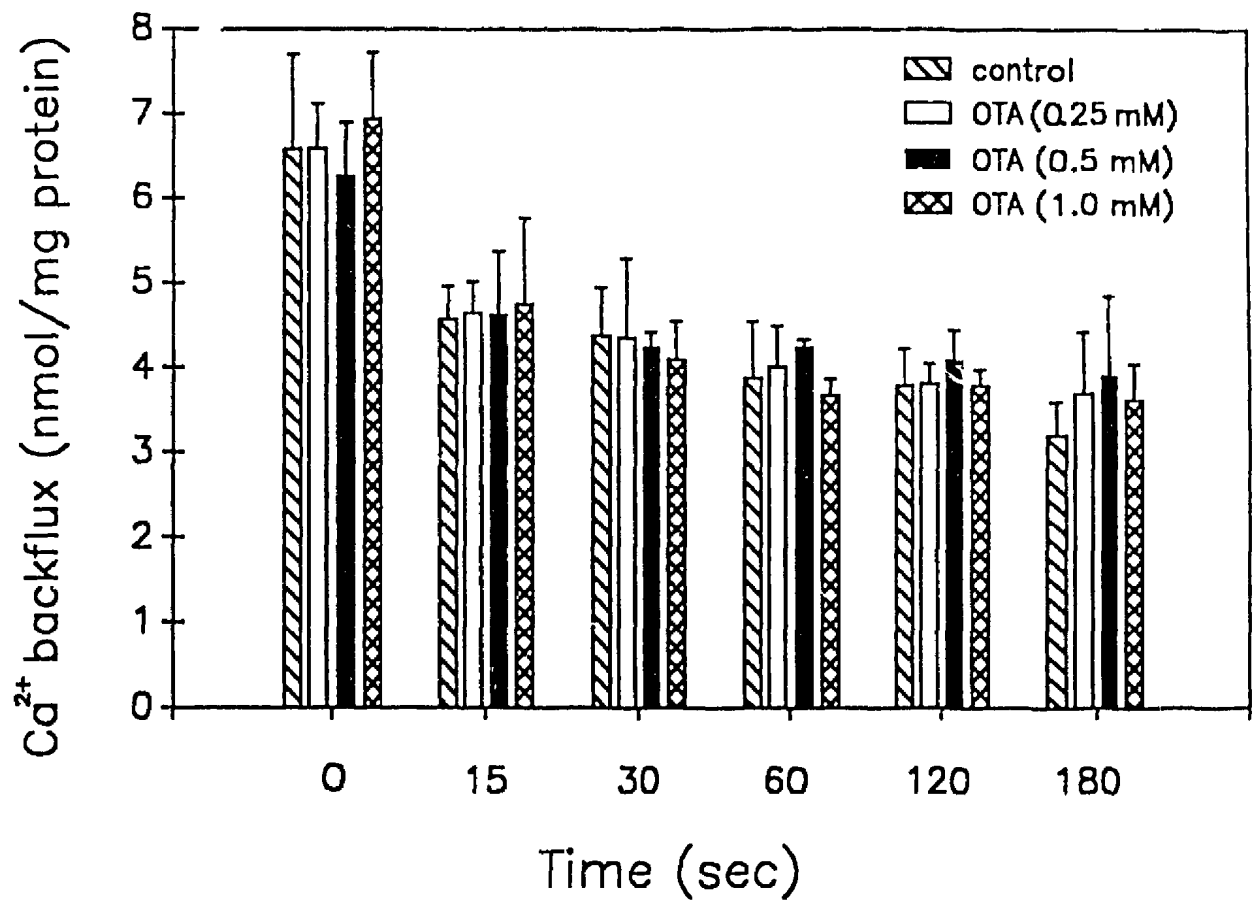
3.3.8 The effect of OTA analogues on renal cortical microsomal calcium pump activity

Different OTA analogues - Ochratoxin B (OTB, de-chloro OTA); Ochratoxin C (OTC, methyl ester of OTA); and Ochratoxin α (OT α , isocoumarin part of OTA), were used in vitro to examine their effects on calcium uptake activity in order to characterize the importance of the chlorine atom, the free carboxyl group and the Phenylalanine moiety, respectively (see Fig. 1 for structures).

Fig. 30 shows the effect of 1 mM OTA, OTB, OTC, and OT α on calcium pump activity in NADPH-preincubated microsomes. OTA gave the highest "stimulation" of calcium uptake, about 7-fold as compared to NADPH alone (from 2.0 to 15.0 nmol/mg protein), followed by OTC (about 2-fold; from 2.0 to 4.4 nmol/mg protein), while OT α and OTB were ineffective.

Fig. 28. Effect of OTA addition on rates of calcium backflux.

Microsomes passively loaded with $^{45}\text{CaCl}_2$ were incubated in KCl-MgCl₂-HEPES-EGTA buffer (pH 7.5) in the presence of various concentrations of OTA. At timed intervals, 0.5 mL aliquots were filtered, washed and bound radioactivity determined as described in 2.2.4.3. Values shown are means \pm S.D. of two experiments, each performed in triplicate.



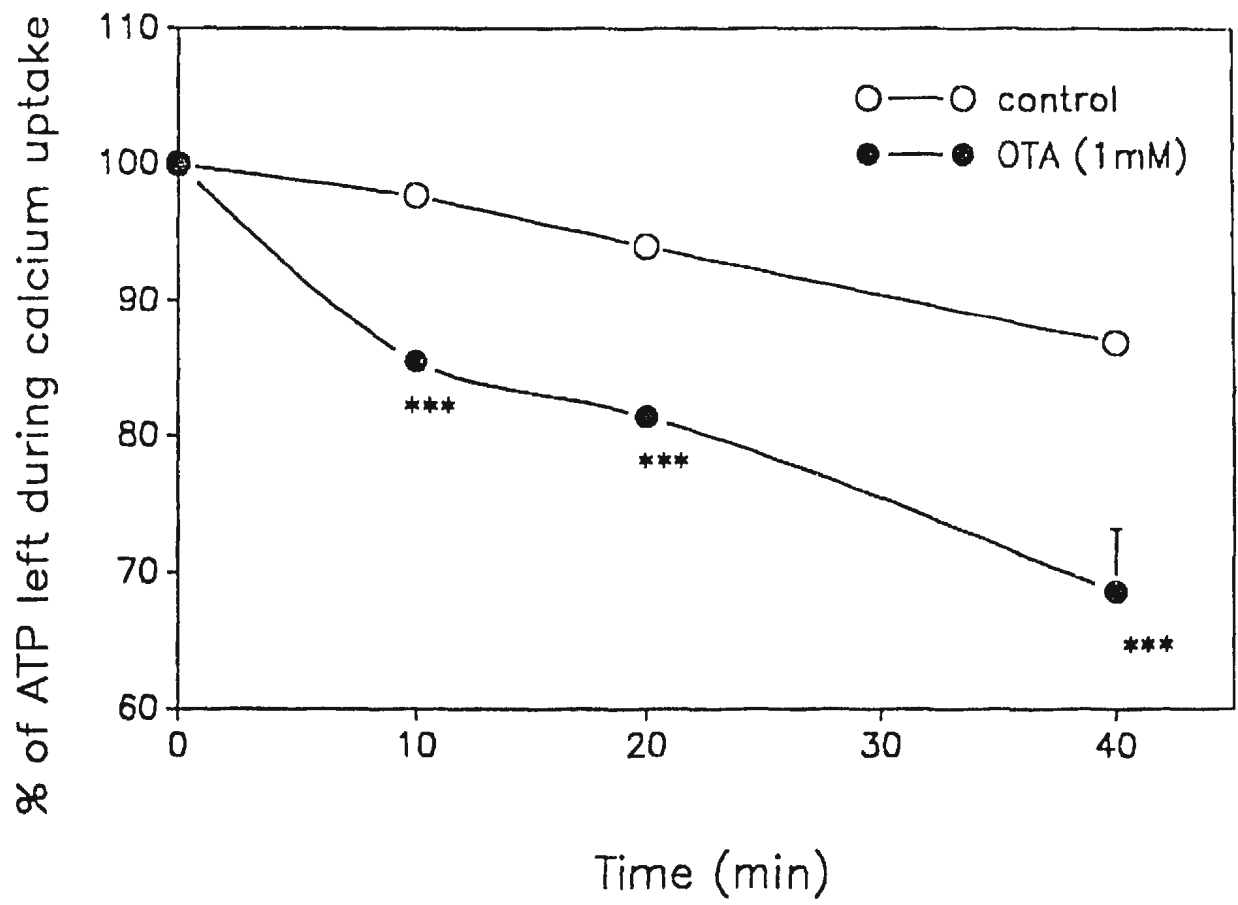
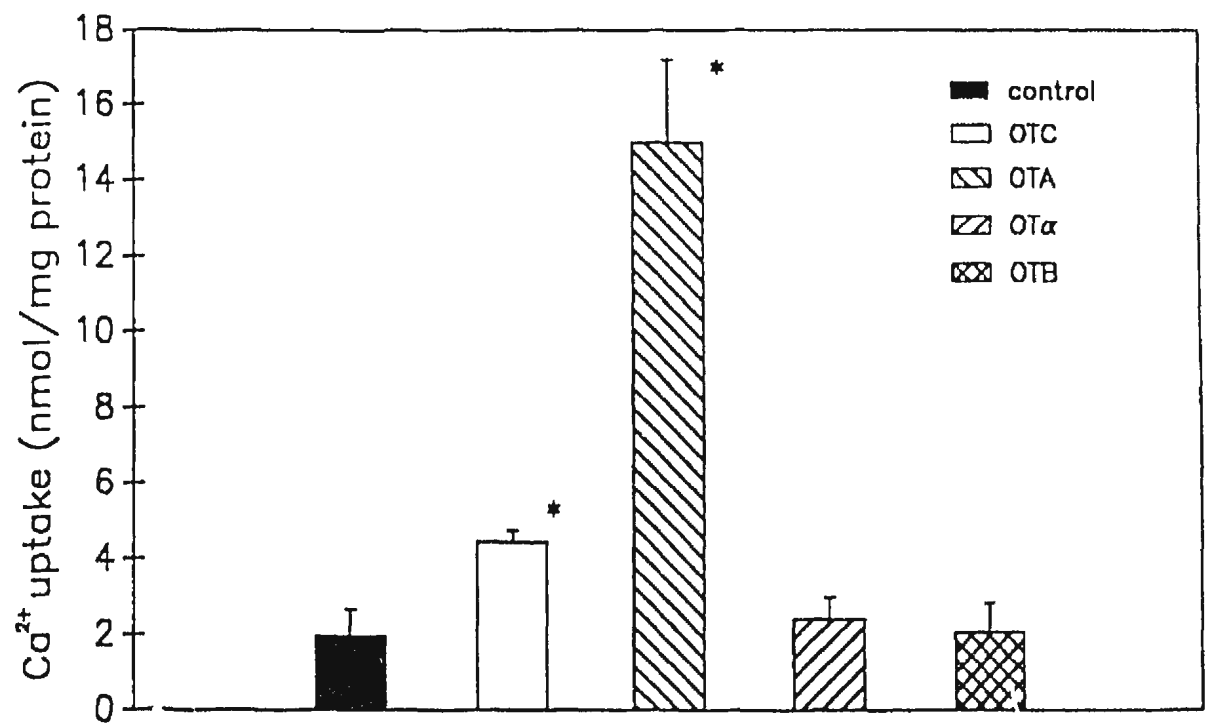


Fig. 29. Consumption of ATP during calcium uptake by microsomes pre-incubated with NADPH in the presence and absence of OTA.

Microsomes were pre-incubated with NADPH \pm OTA (1 mM) for 10 min and the centrifuged microsomes were incubated as described under calcium uptake (Fig. 9) except that $^{45}\text{CaCl}_2$ was omitted. At timed intervals, 0.5 mL aliquots were withdrawn for determination of ATP levels by HPLC as described in 2.2.4.2. Values shown are means \pm S.D. from one experiment performed in quadruplicate.

Fig. 30. The effectiveness of OTA analogues (1mM), OTB, OTC, and OT α in reversing the inhibitory effect of NADPH on renal cortical microsomal calcium uptake.

Experiments were carried out as described in Fig. 15 in the presence of NADPH except that OTB, OTC or OT α were used instead of OTA. Values shown are mean \pm S.D. of two experiments (n=2), each performed in triplicate.



C H A P T E R 4

4. Discussion

4.1 Lipid peroxidation studies

The advent of oxidative stress in vivo often leads to increased levels of antioxidant enzymes which serve to protect the organism/tissue from the deleterious effects of reactive oxygen species and lipid peroxidation products [Barros et al, 1991; Kappus, 1985, Fisher, 1988]. However, the results show that there is neither an induction of any antioxidant enzymes (Table 5 & 6) nor increased levels of lipid peroxides in the kidney subsequent to OTA administration (Table 3 & 4) under a variety of treatment conditions. In fact, significant decreases in some enzyme activities and in MDA levels were observed in kidneys of rats treated 30 min to 2 hr earlier with OTA. Also, in in vitro incubations of renal cortical microsomes with OTA, MDA levels were suppressed (Fig. 8). This suggests that OTA might not produce significant quantities of free radicals in renal microsomes in vitro or in vivo in the kidney during the early stages of treatment. This does not rule out lipid peroxidation as a contributory mechanism to OTA nephrotoxicity under conditions of chronic exposure. However, it appears that lipid peroxidation probably does not play a role during the early stages of OTA

nephrotoxicity. The difference in the values between the two control studies (Table 5) may be due to the varying conditions of the rats from batch to batch, which is also the reason why proper control studies were done in every experiment.

4.2 ER calcium pump studies

The data provide evidence that administration of a single high dose or multiple lower doses of OTA is associated with an increase in renal cortical microsomal ATP-dependent calcium pump activity. The increase in microsomal ATP-dependent calcium pump activity occurs within the first 10 min after OTA administration (i.p.) and lasts for at least 6 hr. A similar increase was also observed 24 hr after a oral dose of OTA. Earlier time points were not examined. The delayed reaction may be due to the fact that OTA is absorbed more slowly when administered orally than intraperitoneally.

It has been shown that calcium uptake by renal cortical slices was substantially enhanced within 5 min of OTA addition [Berndt et al, 1984]. As the endoplasmic reticulum (ER) is considered to represent the major calcium pool inside the cell that maintains calcium homeostasis [Carafoli, 1987], the increase in ER calcium uptake activity observed subsequent to in vivo OTA treatment may be a response to an increase in the cytosolic Ca^{2+} concentration in order to help restore calcium

homeostasis.

Based on the results shown in Fig. 25, it can be said that calcium uptake measured under the incubation conditions is indeed ATP-dependent. When oxalate was present in the medium, calcium uptake was more significant than in its absence (Fig. 26). These results are in good agreement with what Moore et al [1976] observed, and thus provided a reasonable framework for setting up the experimental conditions.

The increase in renal cortical ER ATP-dependent calcium pump activity is the earliest enzymatic change thus far reported in the kidney after OTA administration. De Witt et al [1988] observed a similar increase in renal endoplasmic reticular calcium uptake activity within 4 hr after administration of cisplatin.

However, repeated administration of moderate doses of OTA (4 mg/Kg, i.p., daily for 4 days) or lower dose of OTA (2 mg/Kg, i.p., daily for 8 days) eventually resulted in a decrease in the rate of calcium uptake, which may be due to the dysfunction of calcium ATPase, and could lead to even higher cytosolic calcium concentrations that may further accelerate cell death.

The microsomal calcium pump activity has been shown to be very sensitive to oxidative damage and lipid peroxidation (Waller et al., 1983). Rahimtula et al [1988] have previously

reported that OTA administration to rats increased ethane exhalation (an index of in vivo lipid peroxidation) and inhibited the hepatic microsomal calcium pump activity [Khan et al, 1989]. Also, OTA addition to hepatic microsomes in the presence of NADPH greatly enhanced lipid peroxidation and strongly depressed microsomal calcium uptake. Moreover, a good correlation was observed between inhibition of ATP-dependent calcium uptake and lipid peroxidation as judged by MDA formation [Khan et al, 1989]. The increase in the rate of calcium uptake by renal cortical microsomes was observed soon after OTA dosing (10 min) suggesting that OTA probably does not induce lipid peroxidation in the early stage of its nephrotoxicity. This is in agreement with data showing that no increase in lipid peroxidation was observed (3.2).

In in vitro experiments, OTA itself inhibited the renal microsomal calcium uptake, but partially reversed the inhibition caused by NADPH. Prasad et al [1986] previously observed a similar inhibitory effect of NADPH on rat hepatic microsomal calcium uptake activity and proposed that it is mediated through a cytochrome P-450 pathway, in which an oxygenated cytochrome P-450 complex directly oxidized critical protein thiol groups since GSH was found to reverse the inhibitory effect of NADPH. They suggested that activity of the microsomal calcium pump in vivo is maintained by the cytochrome P-450 system which tends to oxidize sulfhydryls and

thereby decrease activity while the enzyme thio protein disulfide reductase reduces these protein disulfides to reactivate the pump [Holtzman et al, 1989]. On the assumption that, if a substrate is metabolized by a particular isozyme(s) of cytochrome P-450, then its addition should compete for the activated oxygen complex and reverse the NADPH inhibition of the calcium pump, Srivastava et al [1990] concluded that several cytochrome P-450 isozymes, most likely members of the CYP11B and CYP11A family, could be involved in the inhibitory effect. Possible involvement of the CYP11B family in the reversal of the inhibitory effect of NADPH by OTA is consistent with the results of Hietanen et al [1986] who showed that phenobarbital pretreatment of rats induces OTA-4-hydroxylase activity. If OTA serves as a substrate for the particular cytochrome P-450 isozyme(s), it would compete for the active enzyme oxygen complex, thus reducing the latter's ability to induce lipid peroxidation and inactivate the calcium pump, i.e., OTA would seem to activate the calcium pump. The observation that in vitro OTA only increased the V_{max} but not the K_m for calcium uptake (3.3.4.4, Fig. 27b) would be consistent with the above.

In the presence of calcium, addition of [γ - ^{32}P] ATP to hepatic microsomes is followed by rapid formation of the phosphorylated enzyme intermediate [Spamer et al, 1987]. The level of phosphorylated enzyme intermediate has direct

relationship with calcium pump activity. Our results indicated that changes in the rates of renal microsomal calcium uptake correlated with changes in the steady-state levels of the phosphorylated Mg^{2+}/Ca^{2+} -ATPase intermediate. OTA administration which led to an increased rate of ATP-dependent microsomal calcium uptake also showed a higher level of the phosphoenzyme intermediate as compared to control microsomes (Table 7). Thus, it suggests that in vivo/in vitro conditions were affecting the rate of enzyme phosphorylation, which in turn was affecting the calcium uptake level by altering the amount of activated enzyme.

The calcium backflux experiments (Fig. 28), which showed no alteration in the rate of calcium backflux after addition of OTA, indicated that alterations in calcium backflux were not responsible for the change of calcium uptake. Similarly, differences in the rate of ATP consumption can not account for the increased rates of calcium uptake observed after OTA administration in vivo or OTA/NADPH addition in vitro. The K_m for ATP in renal microsomes was found to be 3.1 mM [Moore et al, 1974], thus the increase in calcium uptake in the presence of OTA probably was an under estimation. However, this should not affect the overall stimulating effect of OTA on calcium uptake, since the differences in calcium uptake can be observed within 10 min when more than 4.3 mM of ATP was still available in the incubation (Fig. 29). As proximal cell is

the major site of calcium reabsorption, the altered calcium homeostasis may be the reason why proximal tubule is the primary target site of OTA toxic effect.

The study of the effect of OTA analogues on the calcium pump activity indicated that OTA is the most effective of all the compounds tested in "stimulating" renal cortical calcium uptake. OTC was next with about 1/3 the activity of OTA (3.3.8, Fig. 30). OTB and OT α were very poor in "stimulating" calcium uptake. From this study, it can be inferred that the presence of a free carboxyl group is important since its blockage by esterification, as in OTC, substantially reduced the ability of effecting calcium uptake. The inability of OTB to affect calcium uptake is likely due to the absence of the chlorine atom which is known to alter the pKa of the phenolic hydroxyl [Chu, 1974a]. L-phenylalanine also appears to be a key moiety of OTA since OT α did not show any effect on calcium uptake. How exactly these different groups affect calcium uptake is not known at present.

The inhibitory effect of OTA alone on calcium uptake in vitro could possibly be due to its ability to disrupt the integrity of the microsomal membrane. OTA is known to bind tightly to proteins. Chu [1971] showed that serum albumin bound two equivalents of OTA tightly. Incubation of hepatic or renal microsomes with [3 H]OTA results in the association of a significant amount of protein-bound radioactivity which can

not be removed by repeated washing with trichloroacetic acid and organic solvents [Rahimtula, unpublished data]. Preliminary evidence using ESR spectroscopy also indicates that OTA is able to disturb the ordered structure of hepatic microsomal vesicles containing spin-labelled fatty acids. However, it does not appear that in vivo OTA exerts a significant inhibitory direct effect on renal cortical calcium uptake activity since an increase in this activity was observed after OTA administration.

The data show that in vivo OTA exerts opposite effects on liver and kidney in terms of both microsomal calcium uptake and lipid peroxidation. One possible explanation for this might be that OTA in the presence of cytochrome P-450 might be exerting two effects that oppose each other. As a substrate for cytochrome P-450, OTA would be expected to reduce the steady-state levels of the active cytochrome P-450-oxygen complex and thereby decrease the inactivation of the pump. However, since OTA can also stimulate lipid peroxidation in liver both in vivo and in vitro by a process shown to be mediated by cytochrome P-450 [Omar et al, 1990], the onset of lipid peroxidation would be expected to inhibit the calcium pump activity. In the liver, lipid peroxidation may be dominant leading to an overall inhibition of the calcium pump activity [Khan et al, 1989]. My results show that, under a variety of experimental conditions, OTA does not induce lipid

peroxidation in the renal cortex. It is therefore possible that the increase in calcium uptake activity observed could be due to the inhibitory effect of OTA on the steady-state level of the active cytochrome P-450-oxygen complex.

However, the reason for the inability of OTA to induce lipid peroxidation in the renal cortex or for the involvement of cytochrome P-450 in OTA-dependent changes in renal microsomal calcium uptake remain to be investigated.

C H A P T E R 5

5. Conclusions

- 1) Administration of single high dose or multiple lower doses of OTA to rats resulted in an increase of the renal cortical endoplasmic reticular ATP-dependent calcium pump activity. This increase is the earliest enzymatic change reported (within 10 min) in the kidney after OTA administration.
- 2) Following OTA administration, lipid peroxidation levels and several antioxidant enzyme activities in the kidney were either unaltered or decreased. Thus, lipid peroxidation is not likely to play a significant role during the early stages of OTA nephrotoxicity.
- 3) In vitro preincubation of microsomes with NADPH had a profound inhibitory effect in calcium uptake. Inclusion of OTA was able to partly reverse the inhibition. Addition of OTA alone to cortical microsomes during calcium uptake inhibited the uptake process, although the effect was reversible.
- 4) Changes in the rates of microsomal calcium uptake correlated with changes in the steady-state levels of the phosphorylated Mg^{2+}/Ca^{2+} -ATPase intermediate suggesting that in vivo/in vitro conditions were affecting the rate of enzyme phosphorylation.

- 5) The ability of OTA analogues to restore the inhibitory effect of NADPH on calcium uptake indicated that the presence of a free carboxyl group, the chlorine atom and Phenylalanine moiety on OTA were all essential.

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